

INVESTIGATION OF β AR-DEPENDENT CAMP SIGNALLING IN INDUCED PLURIPOTENT STEM CELL-DERIVED CARDIOMYOCYTES

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ABSTRACT

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) hold great potential for drug testing, cell therapy, and as models of genetic aberrations such as mutations, polymorphisms etc. However, current differentiation protocols do not produce cells which are as mature, both structurally and functionally, as adult cardiomyocytes. In mature cells, β -adrenergic (β AR) pathway regulates the rate and strength of myocyte contraction. This function is strictly regulated by locating β AR in microdomains such as caveolae, and by regulating pools of second messenger cAMP (particularly, via degradation by phosphodiesterases). Also, β AR activity depends on genetic variations within the population. In our study, we assessed β AR signalling in iPSC-CMs, depending on their genetic background and maturation state.

Changes in β AR-dependent cAMP production were assessed in cells with different polymorphic alleles of β AR and at different states of maturation by FRET microscopy and myocyte contractility was measured by the IonOptix technique.

We found that single nucleotide polymorphisms within the β_2 ARs influence receptor sensitivity and desensitisation upon stimulation with isoprenaline, leading to differing myocyte beating rate and cAMP responses.

Three methods of increasing iPSC-CM maturation were explored: peptidomimetic technology, microcontact printing for making cell shape more adult-like and prolonged cell culture.

Treatment with a mimetic peptide of Cav β 2 (a subunit of L-type calcium channel) showed enhanced development of T-tubules, though further investigation remains to be conducted. Microcontact printing positively influenced hiPSC-CMs shape and structural protein alignment, although little change in functional activity was noted. Prolonging time in culture led to an increase in the number of membrane caveolae and increased β_2 AR compartmentation in these microdomains, restricting cytoplasmic β_2 AR-dependent cAMP levels through localised phosphodiesterase activity. Interestingly, limited change of β_1 AR-dependent cAMP activity was observed.

We conclude that β_2 AR signalling pathway compartmentation approaches the adult phenotype in iPSC-CMs with increased time in culture.

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LIST OF ABBREVIATIONS

2D/3D	2-dimentional/3-dimentional
α -actinin	alpha-actinin
AC	Adenylyl cyclase
ADR	Adrenaline
AKAP	Anchoring Kinase A Protein
ANOVA	Analysis Of Variance
AP	Action potential
Arg/R	Arginine
β_1 AR	Beta 1 adrenergic receptor
β_2 AR	Beta 2 adrenergic receptor
β_3 AR	Beta 3 adrenergic receptor
β AR	Beta adrenergic receptor
BIN1	Bridging Integrator 1
bpm	Beats per minute
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
Ca^{2+}	Calcium
cAMP	Adenosine 3',5'-monophosphate
Cav	Caveolae
Cav3	Caveolin-3
Cav β 2	Voltage-gated Ca^{2+} channel β subunit (Cav β)
CDI	Cellular dynamics international
cDNA	Coding deoxyribonucleic acid
CDS	Cell dissociation solution
CFP	Cyan Fluorescent Protein
cGMP	Cyclic guanosine monophosphate
CICR	Calcium-induced-calcium-release
CILO	Cilostamide
CMs	Cardiomyocytes
CRISPR	Clustered regularly interspaced short palindromic repeats
cTnT	Cardiac troponin-T
EB	Embryoid body
ECC	Excitation contraction coupling

EHNA	Erythro-9-(2-hydroxy-3-nonyl)adenine
EHT	Engineered heart tissue
eNOS	Endothelial nitric oxide synthase 3
EPAC	Exchange protein activated by cAMP
ER	Endoplasmic reticulum
FRET	Forster resonance energy transfer
GAF	cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G _i	G-inhibitory
Gln/Q	Glutamine
Glu/E	Glutamic acid
Gly/G	Glycine
GPCRs	G-protein coupled receptors
G _s	G-stimulatory
G _{αi}	Inhibitory alpha subunit of g-protein
G _{αs}	Stimulatory alpha subunit of g-protein
G _{βγ}	Beta-gamma subunit of g-protein
HCN	Hyperpolarization-activated cyclic nucleotide-gated
hESC	Human embryonic stem cell
HuES	Human embryonic stem cell
IBMX	3-isobutyl-1-methylxanthine
ICI	ICI 118,551
I _f	Funny current
IGF-1	Insulin-like growth factor-1
IK ₁	Inward rectifier current
iPSC	Induced pluripotent stem cell
ISO	Isoprenaline
JPH	Junctophilin
K ⁺	Potassium
LTCC	L-type calcium channels
MBCD	Methyl-B-cyclodextrin
MP	Mimetic peptide
mRNA	Messenger RNA
Na ⁺	Sodium

NCX	Na ⁺ —Ca ²⁺ exchanger
NRG1	Neuregulin 1 β
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDMS	Poly-di-methyl-syloxane
PKA	Protein Kinase A
PKB	Protein Kinase B
pTEV	plasmid mTurquoise-Epac1-Venusvenus
PTX	Pertussis toxin
RMPI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
ROLI	Rolipram
RT	Room temperature
RT-QPCR	Quantitative reverse transcription PCR
Ryr	Ryanodine receptors
SCR	Scramble
SEM	Standard error of mean
SERCA	Sarco/endoplasmic reticulum calcium ATPase
sGC	Soluble guanylyl cyclase
SICM	Scanning ion conductance microscopy
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphisms
SR	Sarcoplasmic reticulum
T3	Thyroid hormone triiodothyronine
TEM	Transmission electron microscopy
T-tubules	Transverse tubules
YFP	Yellow fluorescent protein

CHAPTER 1: GENERAL INTRODUCTION

1.1 MYOCARDIAL FUNCTION

1.1.1 The myocardium

The myocardium is the thick middle layer of the heart and is surrounded by an outer layer called the endocardium and a thinner inner layer called the epicardium. The myocardium tissue is highly specialised and composed of striated muscle cells, fibroblasts and cardiac myocytes. Human ventricular cardiomyocytes are tube-like structures measuring 10-15µm in width and 50-100µm in length and are composed of rod-like units forming muscle fibres (myofibrils) (Göktepe et al., 2010; Golob et al., 2014; Sanger et al., 2000). Each myofibril is composed of serially repeating structures, sarcomeres, the fundamental contractile unit of the muscle cell. Each sarcomere is composed of myofilaments, these are long proteins organised into thick filaments (A-band formed of myosin proteins) and thin filaments (I band formed mainly of actin proteins). The overlapping thick and thin myofilaments within the sarcomere of the cell give the characteristic striated appearance (Bray et al., 2008; Golob et al., 2014; Sanger et al., 2000). The sarcomeres have a length ranging from 1.8-2.4µm depending of the degree of contraction and relaxation of the muscle. It's the sliding of the actin and myosin filaments past each other which leads to the formation of "cross-bridges", causing contraction of the heart and allowing force generation (Bray et al., 2008; Geisse et al., 2009; Göktepe et al., 2010) (Fig 1.1).

The cardiomyocyte is surrounded by a plasma membrane called the sarcolemma and functions as a barrier between the extracellular and intracellular contents. Invaginations of the plasma membrane into the cytoplasm of the cardiomyocytes gives rise to structures called T-tubules (Geisse et al., 2009; Göktepe et al., 2010; Scriven et al., 2000). Numerous proteins such as L-type calcium channels (LTCCs), sodium-calcium exchanges, and beta adrenoceptors (βARs) are localised within T-tubules and allow the exchange of various ions between the cardiomyocyte and the extracellular fluid (Scriven et al., 2000).

The contractile power of the myocardium is primarily dependent on cardiac myocytes which account for 70% of the myocardium volume (Zhou & Po, 2016). Their contraction generates pressure to pump blood through the circulatory system. The contraction allows the production of force to propel deoxygenated blood from the body through the right atrium and ventricle to the lungs and oxygenated blood through the left atrium and ventricle to the other organs of the body.

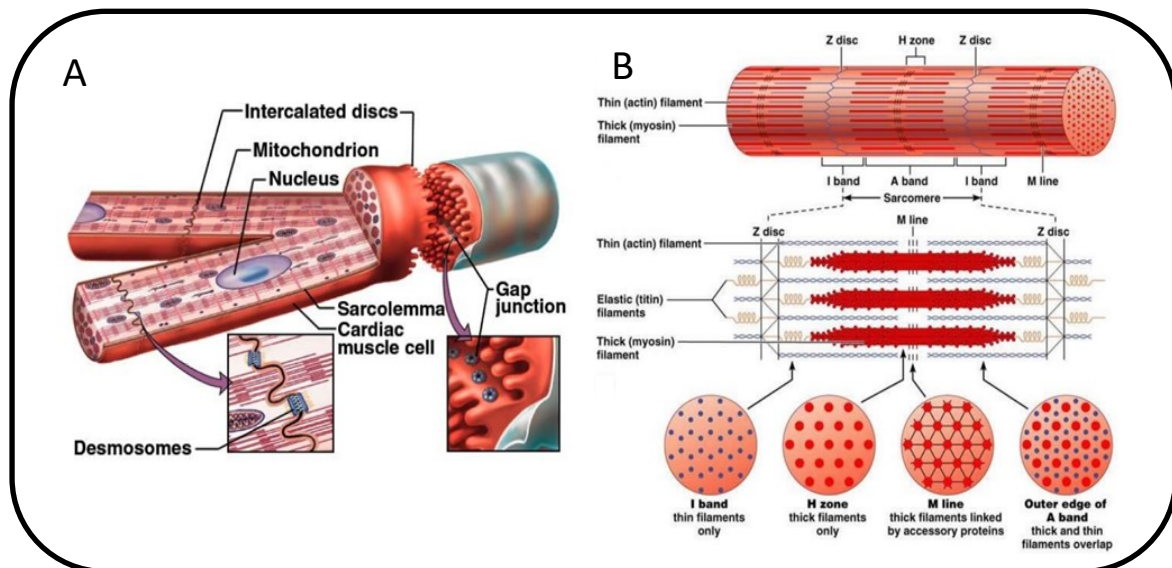


Figure 1. 1 The cardiomyocyte

A) The structure of the cardiomyocyte illustrating the main structural components that are crucial for the function of these cells. B) An illustration of the sarcomere. The main function of cardiomyocytes concerns cardiac contraction. Cardiomyocytes are equipped with bundles of myofibrils that contain myofilaments and represent 45 – 60% of the cell volume (Cardiomyocytes - The Cardio Research Web Project, n.d.).

1.1.2 Excitation contraction coupling

Excitation-contraction coupling is the process by which an electrical stimulation of cardiomyocytes triggers the release of calcium from the sarcoplasmic reticulum and leads to the activation of myocyte contraction by sarcomere shortening. The myocardial action potential occurs in 5 phases and is driven by a series of ion fluxes and molecular events. Pacemaker cardiomyocytes initiate a small depolarisation in surrounding cells causing the fast sodium channels to open and a rapid influx of Na^+

which depolarises the cell membrane potential, phase 0. This influx of ions resulting in the opening of inward chloride channels and a few potassium channels for potassium efflux, and the Na⁺ channels are inactivated. This causes the membrane potential to begin becoming repolarised, but complete repolarisation is prevented by voltage-gated calcium channels causing an influx of Ca²⁺ into the cell. The phenomenon of calcium-induced calcium release is triggered, during which a small increase in Ca²⁺ stimulates the opening of ryanodine channels situated within the sarcoplasmic reticulum membrane. There is a further release of Ca²⁺ store into the cardiomyocyte cytosol which binds to the myofilament protein troponin-C. This causes cardiomyocyte contraction. In phase 2 the efflux of K⁺ and the influx of Ca⁺ are counterbalanced and a plateau phase is maintained. Relaxation occurs when calcium dissociates from the myofilaments via a concentration gradient and the Ca²⁺ is removed from the cytosol. During phase 3, repolarisation occurs as the efflux of K⁺ exceeds the influx of Ca⁺. The final resting membrane potential of the cardiomyocyte is reached, phase 4 (Bers, 2002, 2008).

1.1.3 Modulation of contraction by sympathetic nervous system

The performance of the heart throughout its daily activities is controlled by the cardiac sympathetic and parasympathetic neurons (SN and PSNs) of the nervous system (Bers & Despa, 2009). To perform such function, the peripheral afferents inputs mechanical, metabolic and chemical information and efferent from the central nervous system relay information regarding physiological state, behaviour and emotions to the cervical and thoracic sympathetic ganglia and cardiac parasympathetic ganglia (Armour et al., 1997; James & TN, 1982; Samuels, 2007; Taggart et al., 2006). The autonomic post-ganglionic 'motor' neurons directly innervate the heart and are coupled to the SNs, PSNs and the cardiomyocytes (Hoover et al., 2004). Therefore, these serve as end-effectors of the neuronal network and form the integral brain-heart connections. The entire mammalian heart is innervated by SNs through the epicardium into the myocardial interstitium (Ieda et al., 2007; Kimura et al., 2012).

Acute stresses activate the sympathetic nervous system leading to maximal chronotropic and inotropic activation of the heart. This is an evolutionarily conserved, life-saving response occurring

instantaneously, also known as the 'fight-or-flight' response. The inotropic response is mediated by the β -adrenergic receptors (β ARs). Stimulation of the β ARs leads to an increased production of cAMP which activates protein kinase A (PKA), which phosphorylates various downstream effectors such as the calcium handling proteins; therefore, increasing calcium availability for myocyte contraction (Bers, 2008). The chronotropic response is mediated by the pacemaking activity of the sino-atrial node cells mainly through the action of β_1 AR-dependent cAMP activity and calcium signalling (Difrancesco, 2010). In such situations, the increase in chronotropic and inotropic activity occurs simultaneously in order to maximise cardiac output.

1.2 THE BETA-ADRENERGIC RECEPTORS IN THE MYOCARDIUM

1.2.1 Beta adrenergic receptors

GPCRs are specialised membrane proteins composed of a conserved core structure with an extracellular amino terminus, seven transmembrane α -helices connected by three intracellular and three extracellular loops, and an intracellular carboxyl terminus. These receptors are responsible for the transduction of extracellular signals, such as from hormones and neurotransmitters, chemical stimuli or therapeutic agents. Signalling through GPCRs is vital for regulation of a broad range of physiological processes, such as cell growth, metabolism, sensory perception, and hormonal homeostasis.

The binding of ligands, such as hormones and neurotransmitters, to GPCRs induces a conformational change of the receptor and leads to the GPCR coupling with heterotrimeric G-proteins. This coupling leads to the exchange of G-protein-bound GDP for GTP which induces the dissociation of the G-protein into active G_α and G_β subunits. G_α consist of four subfamilies, G_{α_s} , G_{α_i} , G_{α_q} and $G_{\alpha_{12}}$, which differentiation the cellular signal in order to modulate the activity of various effector molecules and regulate the concentration of second messengers in the cell. For example, the activation of G_{α_s} leads to the activation of adenylyl cyclase (AC) leading to the increase in cAMP concentration. Whereas activation of G_{α_i} inhibits AC activity, reducing cAMP concentration, as well as coupling the receptors to G_i linked pathways and activating extracellular signal-regulated kinases ERK1/2 mitogen-activated protein kinase (MAPK) and PI3K-PKB/AKT pathways. In this way, a variety of different downstream signalling molecules can be activated (Rosenbaum et al., 2009).

1.2.2 Differential roles of beta-adrenergic receptors in the myocardium

One type of specialised GPCRs in particular are the β ARs. Binding of catecholamines to β ARs influences the inotropic, lusitropic and chronotropic activity of the heart. There are three types of β ARs, β_1 AR, β_2 AR, and β_3 AR. β_1 ARs and β_2 AR are highly homologous receptors expressed in the heart, and β_3 ARs are mainly found in adipose with minimal expression in the heart. β_1 ARs comprise 75-80% of cardiac β ARs (Steinberg, 1999) and binds only to G_s (Fig 1.2). It has been shown that selective stimulation of β_1 ARs leads to both positive inotropic and lusitropic effects on cardiac muscle contraction through cAMP production and protein kinase A (PKA) activation (Bers, 2002). The increase in force and speed of contraction is due to an increase in cytosolic Ca^{2+} which occurs due to phosphorylation of L-Type Ca^{2+} channels in the plasma membrane, ryanodine receptors and phospholamban in the sarcoplasmic reticulum. Relaxation is based on an increase in the rate of Ca^{2+} uptake by the SR and a decrease in Ca^{2+} binding to the myofilaments due to phosphorylation of troponin-I proteins.

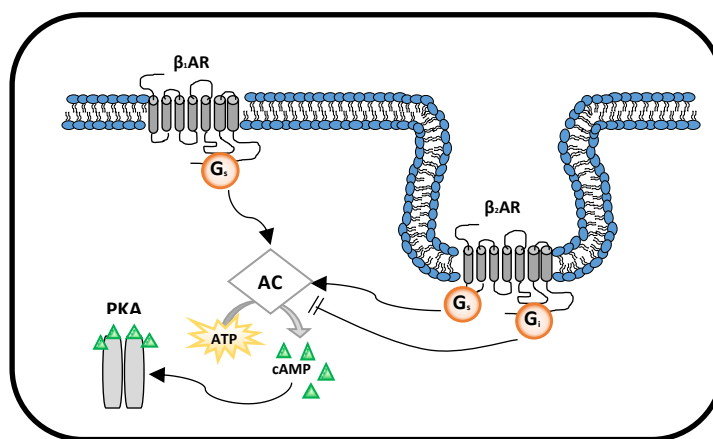


Figure 1. 2 β AR-signalling compartmentation within caveolar microdomains

β_1 ARs and β_2 ARs have distinct signalling pathways and cellular localisations. β_1 ARs are located both in and outside of caveolae, and induce chronotropic and inotropic effects upon stimulation via G_s coupling. β_2 ARs are confined to caveolae and although they can mediate an increased contraction rate via G_s coupling, they can also induce opposing effects by G_i coupling, unlike β_1 ARs.

β_2 AR contribute 20-25% of the cardiac β ARs and can couple to both G_s and G_i proteins, thus the functional responses of β_2 AR can vary quite significantly from those elicited by β_1 ARs. For example, it has been reported that β_2 AR stimulation can lead to a positive inotropic effect, but doesn't produce a positive lusitropic effect observed by β_1 AR. This can be explained by the understanding that β_2 ARs coupling to G_i proteins and can produce a localised cAMP response that is restricted to the regulation of L-type Ca^{2+} channel activity (Balijepalli et al., 2006; Ostrom et al., 2000; Rybin et al., 2000). Therefore, with little or no PKA-dependant phosphorylation of phospholamban or troponin-I proteins, relaxation rate is not increased (Kuschel et al., 1999; MacDougall et al., 2012; Xiao & Lakatta, 1993).

β_3 ARs have been reported to couple to both G_s and G_i proteins. In the human and rodent ventricular myocardium, catecholamine-induced activation of β_3 AR elicits a negative inotropic and positive lusitropic effect by signalling through G_i and the second messenger 3',5'-cyclic guanosine monophosphate (cGMP) (Gauthier et al., 1998; Mongillo et al., 2006). It has been suggested that β_3 AR-cGMP signalling can attenuate prolonged, cardiotoxic β_1 AR-cAMP signalling and protects against pathological hypertrophy and cardiac remodelling (Hammond & Balligand, 2012; Mongillo et al., 2006). One study showed that overexpression of β_3 AR in transgenic mice led to protection of the heart against catecholamine-induced hypertrophy and remodelling through endothelial nitric oxide synthase (eNOS)-soluble guanylyl cyclase (sGC)-cGMP signalling pathway (Belge et al., 2014). This thesis focuses on cAMP activity through β_1 AR and β_2 AR, and so discussion of β_3 AR-cGMP activity will be limited.

β_1 AR and β_2 AR are found to be located in different membrane regions, which raises the question of whether cAMP produced by stimulation of receptors located in different membrane domains play an equal role in regulating the various downstream targets. β_1 AR have a widespread distribution and are located in all fractions of the plasma membrane, including caveolar and non-caveolae lipid rafts, as well as non-lipid raft fractions. Interestingly, G_s proteins, which couple with β_1 ARs to AC, are also found to be distributed in a similar pattern. In addition, AC5 and AC6, the most common AC isoforms in

cardiac tissue, are also found to be expressed in both caveolar and non-caveolar fractions (Nikolaev et al., 2006, 2010). There is evidence suggesting that AC5/6 interacts directly with membrane Cav3, thus explaining the localisation of AC5/6 to caveolar fractions (Harvey & Calaghan, 2012). On the other hand, β_2 ARs are mainly found within the caveolin-enriched fractions of the plasma membrane, together with G_s , G_i and AC5/6. FRET-based studies using a type II PKA-based probe show that β_2 AR activation not only elicits localised cAMP responses (MacDougall et al., 2012), but also produces a response which can be detected globally using cytosolic sensors (MacDougall et al., 2012; Nikolaev et al., 2006; Wright et al., 2014). This leads to the assumption that although β_2 AR-produced cAMP may not be restricted, it doesn't readily diffuse throughout the cell (Nikolaev et al., 2010; Wright et al., 2014).

The effect caveolae and/or lipid rafts have on producing compartmentalised responses has been investigated through treatment with M β CD to deplete membrane cholesterol from the membrane bilayer. It has been shown that cholesterol depletion or Cav3 knockdown with siRNA doesn't change the effect maximal β_1 AR activation has on contraction or L-type Ca^{2+} channels in adult ventricular myocytes. Furthermore, treatment of adult cardiac myocytes with M β CD showed to enhance the inotropic response to β_1 AR stimulation (Calaghan & White, 2006; Agarwal et al., 2011). Through the use of either membrane-localised FRET biosensors targeting type II PKA locations or cytoplasmic sensors targeting type 2 exchange protein activated by cAMP (Epac2-cAMP) global and localised cAMP changes have been measured. It revealed that treatment with M β CD increased local cAMP responses within the PKA signalling domain, but not the global cAMP responses (MacDougall et al., 2012; Agarwal et al., 2011). Interestingly, disrupting caveolar function with M β CD has shown to cause a redistribution of β_2 ARs, resulting in a cAMP response that propagates throughout the cell (Nikolaev et al., 2006).

1.2.3 Biased agonism of β_2 AR

There is increasing evidence for the ability of the β_2 AR to interact with a specific subset of G-protein which attributes to the differing physiological properties of the β_2 AR from β_1 AR. In addition to interacting with G_s proteins, β_2 AR can also couple with G_i proteins, thus having a major influence of cardiomyocyte contractility. The ability of β_2 AR to switch between coupling from G_s to G_i is a process known as biased agonism (or stimulus trafficking). This has been shown to occur based on post-translational modifications which influence PKA-mediated β_2 AR phosphorylation (Daaka et al., 1997).

Agonist-mediated activation of the β_2 AR and its GPCR-subtype coupling is thought to occur in a dose-dependent manner. For example, previous literature on transgenic mice over expression β_2 AR showed to have a positive inotropic effect through β_2 AR- G_s upon receptor stimulation with low adrenaline concentrations. However, with increasing adrenaline concentration, there was a switch of β_2 AR coupling from G_s to G_i , eliciting negative inotropic effects (Heubach et al., 2004). This suggests that catecholaminergic stimulation at higher physiological levels activates an alternative signalling pathway which triggers the switch of β_2 AR coupling from G_s to G_i ; and by doing so, further positive inotropy is inhibited. Biased agonism can depress cardiac contractility and therefore would be favourable at higher concentration. In this way, it proves an important method for preventing overstimulation of the cells by the β AR and also protecting against β_1 AR-dependent catecholamine-induced cardiotoxicity.

Previous work by Zhu et al. (2001) reported that stimulation of the β_1 AR in a β_2 AR-knock-out mouse led to cardiomyocyte apoptosis. In addition, they reported that β_2 AR stimulation, on the other hand, promoted anti-apoptotic effect to a certain extent (Zhu et al., 2001). Pertussis toxin (PTX) is commonly used as a treatment for inhibition G_i and has been reported to enhance cellular apoptosis upon β AR stimulation (Communal et al., 1999). Foester et al. 2003 further investigated the pleiotropic property of β_2 ARs by inactivating the cardiac $G_{i\alpha}$ subunit by gene deletions. They observed reduced survival of homozygous knockout mice, and heterozygous mice were prone to heart failure and cardiac

hypertrophy (Foerster et al., 2003). This highlights the essential role β_2 AR- G_i interactions in cardiac protection against chronic adrenergic receptor stimulation, and essentially working towards providing prolonged survival and delaying cardiac pathology.

1.2.4 Biased agonism in the clinical setting

In response to external stimuli, the contractility of the heart can increase dramatically as and when required. However, at times of physiological stress, levels of catecholamines can be significantly elevated; and unlike the healthy myocardium, a chronically failing heart can become insensitive to prolonged periods of receptor stimulation. This is a result of β_1 AR down regulation and, although β_2 ARs level are maintained, there is β_2 AR- G_s uncoupling and increased β_2 AR- G_i coupling.

Takotsubo cardiomyopathy is the apical ballooning an acute yet severe heart failure syndrome in which this phenomenon of biased agonism is observed. It is shown to mimic the symptoms of acute myocardial infarction and has been reported to occur shortly after periods of stress, hence also termed “stress cardiomyopathy” (Sattler et al., 2018; Ghadri et al., 2018). Takotsubo cardiomyopathy was first described in 1990 in Japan, where the shape of the left ventricle (LV), with an akinetic apex and hyperkinetic basal segments, was compared to a Japanese Octopus pot, or takotsubo (Sato et al., 1990).

The pathophysiological mechanism of Takotsubo syndrome are still unclear, but it has been proposed that excessive levels of circulating adrenaline may be an important underlying factor (Wittstein et al., 2005). In most cases Takotsubo patients make a full recovery, however, significant mortality has been documented in the early stages of onset. The observed reversibility of Takotsubo has sparked interest in understanding the potential protective, cardiodepressive mechanism which provides a beneficial physiological function (Wittstein et al., 2005).

Murine rat models have been created by adrenaline infusion to mimic this clinical syndrome and examine the involvement of the β ARs to show the reversible, negative inotropism in the apex. As discussed earlier, the β_1 ARs only signal via the G_s pathway, whereas the pleiotropic β_2 ARs can signal via G_s or G_i , thus limiting toxic G_s activity and reversing its contractile effect. This effect is observed to a greater extent in the apex due to the increased sensitivity of the β ARs and a greater ratio of β_2 AR to β_1 AR (Paur et al., 2012). This *in vivo* model has proved to be a useful model to explore the role of β_2 AR- G_i interaction and has been extensively been used to study the cardioprotective nature of Takotsubo syndrome. It has also been previously demonstrated by Paur *et al.* that the cardiodepressive effects are induced by high doses of adrenaline (Paur et al., 2012). Thus, with the activation of G_i -signalling in the presence of PTX, this protective effect is abolished and there was a high level of arrhythmic mortality observed. Therefore, the β_2 AR-stimulus trafficking towards G_i is protective against catecholamine-induced toxicity.

1.2.5 Localisation of β_2 AR in TT

The sympathetic control of the myocardial function is mainly driven by the β_1 AR and β_2 AR pathways. However, there is accumulating evidence that signalling through these two receptors results in differential physiological responses. This has been made possible through the use of scanning ion conductance microscopy (SICM) in combination with FRET. SICM allows non-contact, live imaging of cell membranes with high resolution and stability; moreover, it allows the application of molecules and reagents to localised areas of the cell. SICM together with FRET microscopy, allowed real-time measurements of cAMP activity upon stimulation of receptors in specific regions of the cell membrane. With the use of FRET alone, it was found that whole cell β_1 AR activation leads to the stimulation of cAMP-PKA mediated phosphorylation of phospholamban and other contractile proteins (Chen-Izu et al., 2000). Excessive/prolonged β_1 AR stimulation can induce hypertrophy progressive hypertrophy and heart failure in β_1 AR transgenic mice and can promote cardiomyocyte apoptosis

(Engelhardt et al., 1999). Nikolaev et al showed through the use of scanning ion conductance microscopy (SICM) in combination with FRET that local stimulation of β_1 AR lead to whole-cell cAMP propagation, whereas local stimulation of β_2 AR had only locally confined responses (Nikolaev et al., 2010; Schobesberger et al., 2017). Further to this they show that although β_2 ARs are present on the crest as well as in t-tubules, cAMP was only produced from β_2 AR when stimulated in the T-tubules (Calaghan et al., 2008; Zakhary et al., 2000). The localised cAMP response of β_2 AR explains the limited effect it has on myocyte contraction and relaxation. Stimulation of both β ARs led to an increase in LTCC and enhanced contractile strength, but only β_1 AR stimulation led to phosphorylation of phospholamban, troponin-I and troponin-C proteins leading to enhanced muscle relaxation (Kaumann et al., 1999). Using the cell-attached patch-clamp technique, Chen-Izu et al also show that when either β_1 AR or β_2 AR are stimulated with agonist within the patch membrane, both activated LTCCs within the patch (Chen-Izu et al., 2000). However, when agonist was applied outside the patch membrane, only β_1 AR lead to channel activation; deducing that β_1 AR signalling is global and can diffuse to activate channels throughout the cytosol, however, β_2 AR signalling is only local to the cell membrane (Gorelik et al., 2006). In heart failure, as a result of changes in cardiomyocyte structure, β_2 AR redistribute to the cell surface and responses were no longer exclusively detected in the t-tubules, but were also detected in the crest region. This is due to the uncoupling of the β_2 ARs from localised PKA molecules, thus the compartmentation of the β_2 AR-cAMP signalling is lost (Schobesberger et al., 2017; Gorelik et al., 2013; Zaccolo, 2011). The delocalisation of β_2 AR in failing cells also leads to increased phosphorylation activity upon receptor stimulation; which is potentially due t-tubule disorganisation during heart failure (di Benedetto et al, 2008).

1.2.6 Haplotypes of the β_2 AR

The human β_2 AR is located on chromosome 5(q31-32) and is an intronless gene. There are many single nucleotide polymorphisms (SNPs) identified in the human population. They are randomly located throughout the receptor gene and are suggested to influence receptor expression, agonist binding properties and receptor desensitisation. With increasing evidence for the influence these genetic variants have on the adrenergic response and the downstream signal transduction pathways (Evans & McLeod, 2003), β_2 ARs are an interesting pharmacological target for modifying cardiovascular function.

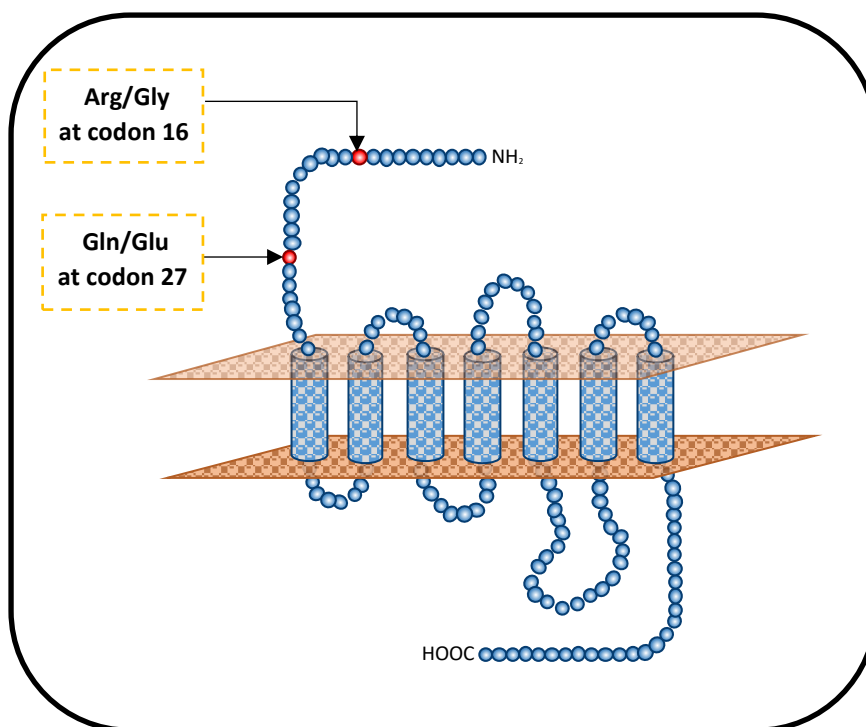


Figure 1. 3 Structure of the human β_2 AR

The human β_2 AR is composed of 413 amino acids, 3 intracellular and extracellular loops, and 7 transmembrane domains. Shown in red are the locations of the two most common single nucleotide polymorphisms (SNPs) in the human DNA sequence which result in altered amino acids at codon 16 and codon 27 of the receptor.

The most common polymorphisms of the human β_2 AR are situated at the amino acid position 16 and 27, this leads to an Arg-to-Gly and a Gln-to-Glu amino acid change respectively (Fig 1.3). These variants in particular have been associated with influencing cardiac physiology. In addition, linkage

disequilibrium of these alleles results in the frequency of these variants to differ between different ethnic backgrounds. The reported allele frequencies for Gly-16 were 0.61, 0.50 and 0.40 and for Gln-27 were 0.57, 0.73 and 0.80 in white, black and Asian populations respectively (Liggett, 2000).

In vivo experiments have previously been set up to study the functional effects of these genetic variants. One study investigated β_2 AR-agonist-mediated vascular desensitisation by infusion of isoprenaline in individuals with variable amino acids at position 26 and 27 of the β_2 AR. They showed that patients homozygous for Arg16 had a higher rate of agonist-mediated desensitisation, indicating that the reduced responsiveness may be involved in a cardioprotective process. Whereas those patients homozygous for Glu27 showed enhanced agonist-mediated responsiveness (Dishy et al., 2001).

The influence of these haplotypes on β_2 AR desensitisation was also investigated by Bruck *et al.* who measured the change in heart rate and contractility after an intravenous treatment of terebualine *in vivo*. Similarly, to the study conducted by Dishy et al., (2001), Bruck *et al.* observed slower desensitisation in individuals who were homozygous for the Glu27 variant (Bruck et al., 2003). Thus, supporting the hypothesis that the Glu27 variant is responsible for enhanced β_2 AR-agonist-mediated due to resistance to desensitisation.

Although SNPs within the β_2 AR have shown to alter phenotype and can greatly influence cardiac health, studying only individual SNPs have a poor predictive power as pharmacogenetic loci. This is mainly due to the theory that unique interactions which exist between the SNPs themselves can be missed.

Most commonly *in vivo* experiments have been conducted to observe the properties of the β_2 AR haplotypes. However, more recently, cardiomyocytes from human embryonic stem cells have been developed to model an array of phenotypes which is also proving beneficial as they can more easily

be genetically manipulated. The Denning group in Nottingham have genetically manipulated one such hESC line, HuES7, using CRISPR/Cas9 technology to develop cell lines which stably express the β_2 AR variants of amino acid 16 and 27 (where Arg16Gly is (R/G) and is Glu27Gln (E/Q)) resulting in the GE, GQ, RE and RQ haplotypes (Kondrashov et al., 2020). This tool will allow further investigation into understanding the influence of the β_2 AR haplotypes on receptor activity and cAMP handling properties.

1.3 cAMP SIGNALLING IN CARDIOMYOCYTES

1.3.1 cAMP synthesis and signalling pathway

Adenosine 3',5'-monophosphate (cAMP) is a small diffusible molecule which acts as an intracellular messenger and regulates a wide range of cellular and biological functions, such as gene transcription, cell metabolism and development, among other specialised functions depending on cell type. The cAMP pathway is triggered by the binding of a hormone which activates GPCRs. This leads to adenylyl cyclase (AC) activation which catalyses the conversion of ATP to cAMP. This cAMP then binds to and activates specific intracellular effectors, such as protein kinase A (PKA) or cAMP-dependent exchange proteins (Epac); which are the two main cAMP effectors which mediate downstream signalling. In addition, cAMP can also activate cyclic nucleotide-gated ion channels (CNGC) and POPDC proteins (Beavo and Brunton, 2002).

Most cells express various types of GPCRs which signal via cAMP, and each cell contains numerous effectors which can be phosphorylated by PKA; thus, a great degree of sophistication in response to extracellular stimuli is required to obtain coordinated behaviour for the desired cell output (Beavo et al., 2006; Conti & Beavo, 2007). One such way of maintaining specificity is by having a multiplicity of isoforms/variants for each of the molecular components involved in the signalling pathway. In this way, isoforms can be unique to specific pathways. Alternatively, signalling components can be spatially confined to subcellular locations, thus restricting regulatory mechanisms and raising the concept of cellular compartmentation (Francis et al., 2001). This new perspective of locally regulated signal propagation is beginning to unravel novel insights into pathophysiological mechanisms and potential avenues for therapeutic interventions. In addition to the protein components of the signalling pathway, spatial confinement also includes cAMP itself. This was initially demonstrated in cardiomyocytes (Zacclo, 2006).

In the heart, stimulation of both β ARs and prostaglandin receptors with isoprenaline and prostacyclin respectively results in a similar increase in cAMP through the G-stimulatory pathway. However, only cAMP generated from β ARs activation increases myocyte contraction but not by the activation of the prostaglandin receptor. This was further studied using real-time cAMP analysis which showed that both these receptors generate a spatially distinct pool of cAMP which activate different subsets of PKA enzymes, thus phosphorylating different downstream effectors. It is the influence of catecholaminergic stimulation of the β ARs which occurs specifically in pools of the membrane bound PKA-regulatory domain II (PKA-II) which results in an increase in phosphorylation of contractile proteins of the myocyte. Prostacyclin stimulation of prostaglandins stimulates the cytosolic PKA-RI domain which exist in a different pool, thus effecting targets of a different signalling pathway (Mongillo et al. 2004).

1.3.2 cAMP degradation and compartmentation by PDEs

The amplitude, duration and localisation of cyclic nucleotides relies on the fine balance between cyclic nucleotide synthesis and, subsequently, its degradation by phosphodiesterases (PDEs). PDEs are a highly diverse superfamily of enzymes that play a central role in lowering cyclic nucleotide levels; thus, obtaining control of cAMP concentrations throughout the cell and is therefore has a major influence on cAMP compartmentation. The PDE superfamily are composed of 11 families. PDE1, 2, 3, 10, 11 have dual specificity and can hydrolyse both cAMP and cGMP. PDE 4, 7, 8 families specifically target cAMP and PDE5, 6, 9 hydrolyse cGMP. Different translational start sites and alternative mRNA splicing gives rise to over 100 different PDE isoforms which vary in primary structure, catalytic properties and affinities for cAMP and cGMP. These variable enzymatic properties and localisation can directly shape, separate and modulated cyclic nucleotide to specific subcellular microdomains. PDEs have a conserved catalytic domain with 25-52% amino acid sequence homology and the regulatory N-terminal domain can be highly variable, and is used to characterise each family and isoform variant.

The N-terminal is also responsible for regulating enzyme dimerization, binding of regulatory molecules, phosphorylation sites targeted by protein kinases and membrane-associated domains for localisation of PDEs to specific subcellular compartments (Conti et al., 2014). Furthermore, individual cell types uniquely express different combinations of these PDE isoform variants and therefore have different subcellular localisations and functional activity (Kim & Kass, 2017). This local degradation of cAMP and cGMP by PDEs allows signalling to be compartmentalised within defined pools (Leroy et al., 2008). Individual PDEs can also be functionally coupled to GPCRs and degrade cAMP selectively to a given stimulus (Mongillo et al., 2004).

This specificity is critical to achieve specific physiological responses (Hambleton et al., 2005; Richter et al., 2001) and their dysregulation, disrupted targeting or blunted responsiveness to stimulation can contribute to pathological remodelling, muscle dysfunction, metabolic abnormalities and poor cell survival (Beavo et al., 2006). Changes in the expression or activity of certain PDEs can lead to abnormal cAMP handling and has been noted in various cardiovascular diseases; therefore, PDEs can be important targets for pharmacological interventions, involving either the synthesis or suppression of some PDE families or specific isoforms (Fig 1.4).

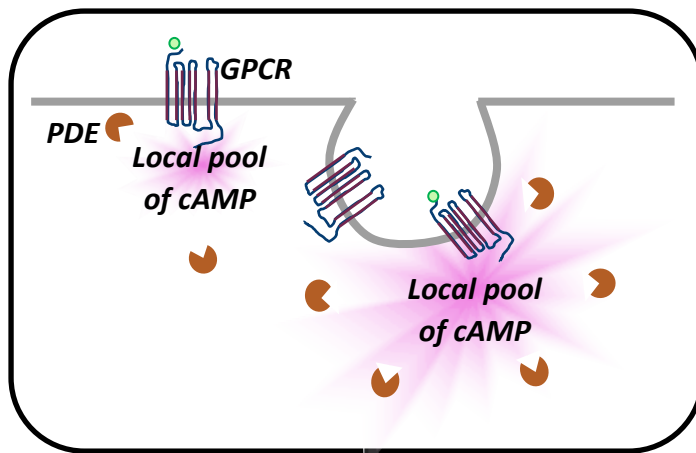


Figure 1. 4 Phosphodiesterases compartmentalise cAMP pools

Phosphodiesterase's dynamically control cAMP molecules in various subcellular regions by functioning as a barrier against cAMP diffusion, thus are capable to generation compartments of high and low cAMP concentrations in different cellular domains, and ultimately control the activity of cAMP effectors protein kinase A (PKA) and exchange protein activated by cAMP (EPAC).

PDE2, 3, and 4 are most commonly involved with cAMP degradation in the cardiomyocyte with some variability between species. In mouse and rat, PDE4 is responsible for 60% of cAMP hydrolysis (Beavo et al., 2006). PDE3 and PDE4 together contribute to 90% of cAMP degradation in rat cardiomyocytes (Mongillo et al., 2006). Human cardiomyocytes show PDE3 plays an equal role in cAMP hydrolysis to PDE4 (Beavo et al., 2006; Mongillo et al., 2004).

1.3.2.1 PDE2

PDE2 has dual specificity for both cAMP and cGMP and the splice variants produced from a single gene gives rise to three gene products, PDE2A 1-3. These enzymes are assembled as homodimers where each monomer possesses two regulatory GAF domains (GAF = cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) to which cGMP binds, increasing its catalytic activity by to up 30-fold. They are stimulated by the binding of cGMP to the GAF domain. PDE2 is found in the cytosol and is also associated with functional membrane structures, such as the plasma membrane, sarcoplasmic

reticulum and the nuclear envelope (Lugnier, 2006). The role of PDE2 at the plasma membrane is shown to be essential to regulate cardiac LTCC activity when cGMP levels are elevated (Fichmeister et al., 2005). In the heart, it is thought that PDE2 reduces cAMP levels in response to cGMP production. Previous studies performed using FRET microscopy showed that in neonatal cardiomyocytes a negative feedback mechanism involving β_3 AR and eNOS/sGC/cGMP signalling pathway led to PDE2 activation. This counteracted the increase in cAMP and the positive inotropic response after β AR stimulation (Mongillo et al., 2006). It has previously been suggested that PDE2 accounts for only a small fraction of total cAMP hydrolysing activity in the myocardium (Beavo et al., 2006; Mongillo et al., 2004); however, in the presence of cGMP, PDE2 still plays a vital part in regulating specific pools of cAMP.

1.3.2.2 PDE3

The PDE3 family also have dual-specificity for cAMP and cGMP; however, in contrast to PDE2, PDE3 activity is inhibited by the interaction with cGMP. Although it's a dual substrate PDE, it hydrolyses cGMP with a lower $V_{\max}$ than cAMP; therefore, in theory, cGMP acts as an inhibitor of cAMP hydrolysis and so, localised production of cGMP leads to restricted increases in cAMP concentrations during β AR stimulation and promotes increased cardiac function (Stangherlin et al., 2011). It has two isoforms, PDE3A/B, where 3A is shown to be the predominant variant in mammalian hearts (Sun et al., 2007; Kim et al., 2017). The alternative splice variants and post-translational modifications give rise to isoforms with different N-terminal lengths with single association domains and also PKA and PKB phosphorylation sites (Wechsler et al., 2002; Hambleton et al., 2005). The latter regions are essential for regulating the feedback mechanisms to control cAMP levels in cardiomyocytes (Rochais et al., 2004), thus allowing preferential targeting of PDE3 in specific cellular domains and ultimately compartmentation of cAMP. In the human myocardium, PDE3 isoforms are found to localise in cytosolic and membrane fractions. cAMP metabolism in these fractions is regulated in an independent

manner and changes in cAMP content have been found to correlate with changes in different physiological parameters, e.g. Ca^{2+} homeostasis and contractility (Rybin et al., 2000; El et al., 1980).

1.3.2.3 PDE4

Unlike PDE2 and PDE3, PDE4 is cAMP-specific and is not regulated by cGMP. It is encoded by four genes: PDE4A/B/C/D with over 20 isoforms, although PDE4A/B/D are expressed in the myocardium (Richter et al., 2011). Previous literature suggest PDE4 plays a significant role in cAMP hydrolysis. For example, upon the use of 1m of cAMP on murine myocytes, PDE contributed up to 60% to total cAMP-hydrolysis (Molina et al., 2012). It has been shown that PDE4 has no effect on basal cAMP levels in adult rat and mice ventricular myocytes but its activity becomes crucial to hydrolyse cAMP produced upon β AR stimulation. PDE4 also shows to play in important role in controlling PKA phosphorylation of key ECC proteins, such as LTCC, RyR2 and PLB. Hence, inhibition of PDE4 greatly enhances the inotropic effects of β AR stimulation. When ventricular myocytes of mice lacking PDE4B were challenged with isoprenaline, enhanced L-type channel activity was detected, leading to augmented calcium transients and shortening, as well as increased calcium release events resulting in ventricular arrhythmias under β -AR stimulation (Leroy et al., 2011). The disruption to PDE4D activity in mice led to delayed after depolarisation, exercise induced arrhythmias, late-onset cardiomyopathy and a significantly increased mortality rate after myocardial infarction (Knight et al., 2012). These results emphasize the importance of strategically located phosphodiesterase isoforms for adequate control of cardiac ECC and arrhythmias.

Although there is a growing amount of experimental evidence supporting the critical role PDE4 plays in rodent hearts, the physiological effect of PDE4 in the human heart may vary. It has been suggested in one study that in human myocytes, PDE4 seems to have less of a role, contributing to <10% of cAMP-hydrolysing activity (Richter et al., 2011). Another study reports that PDE4 represents a large

proportion of cAMP hydrolysing activity, particularly in human atrial tissue, and is also essential for the regulation of isoprenaline-induced cAMP production. Furthermore, it was detected that patients with atrial fibrillation had reduced PDE4D functionality (Molina et al., 2012).

Although PDE4A/B isoform activity is minimally detected in rodents and human myocardium and little is actually known about their roles in cAMP compartmentation, there is a growing body of literature of PDE4D isoform. Nevertheless, the role of PDE4 in the human heart needs to be further investigated.

1.3.3 Caveolae compartments in cardiomyocytes

The originally proposed fluid mosaic model of the lipid bilayer indicated that membrane proteins are freely diffusible through the cell membrane (Singer & Nicolson, 1972) therefore leading to the idea that signal transduction occurs through a process of random collision of proteins involved (Tolkovsky & Levitzki, 1978). However, the low density of proteins makes their theory unfeasible for obtaining efficient and accurate responses. More recently, Bethani et al demonstrated that specific mechanisms exist in favour of restricted movement of membrane proteins (Bethani et al., 2010). In this way the plasma membrane can be separated into distinct microdomains so that signalling molecules can be concentrated with limited diffusion (Fig 1.4).

Jacobson et al. described these specialised lipid rafts called caveolae as flask-shaped invaginations of the plasma membrane, 50-100nm in diameter, concentrated with cholesterol, sphingolipids and phospholipids (Jacobson et al., 2007) (Fig 1.5). Caveolae have been described to take on many roles: as signalling platforms, in endocytosis, transcytosis, membrane lipid homeostasis and responsible for buffering mechanical membrane stress (Balijepalli et al., 2006; Nichols et al., 2010; Rybin et al., 2000; Xiang et al., 2002).

Caveolae are also rich in cavin and caveolin proteins. Caveolins are 18-22kDa membrane scaffolding proteins with a hairpin-like configuration. These are found at the intracellular face of the plasma membrane and can assemble as oligomeric complexes, resulting in the formation of the characteristic flask-shape. There are 3 caveolin isoforms, Cav1/2/3. Cav3 is muscle-specific (Patron & Way, 1995) and is the predominant isoform found in cardiac myocytes, although the expression of all three types has been previously reported (Head et al., 2015). Caveolae formation is initiated by the assembly of caveolins into small oligomers in the endoplasmic reticulum (ER), which are then transported to the Golgi apparatus where they are assembled with cholesterol and other oligomers. This complex is then exported to the plasma membrane as an “intact protein coat”. Once at the plasma membrane, cavins, 50kDa adapter proteins, then oligomerise and assemble to form an outer coat complex. This leads to remodelling of the membrane into caveolae (Busija et al., 2017).

Membrane fractionation studies have illustrated that certain proteins are concentrated in these lipid rafts whereas others are specifically excluded (Gestel et al. 2005). The mechanism which regulates protein targeting to or excluded from lipid raft domains of the plasma membrane are not fully understood, but some proteins have a greater affinity for an ordered membrane environment (Lorent et al., 2017). Interestingly, certain proteins, like GPCRs, are able to bind to the cholesterol and other lipid components of rafts thus explaining their preference for these microdomains (Gahbauer et al., 2016). The binding of agonists can also alter receptor affinity for cholesterol thus inducing the translocation of receptors between these domains. In addition, post-translational modification, acylation and protein-protein interactions (particularly with caveolins) also play important roles in targeting proteins to lipid rafts (Lorent et al., 2017).

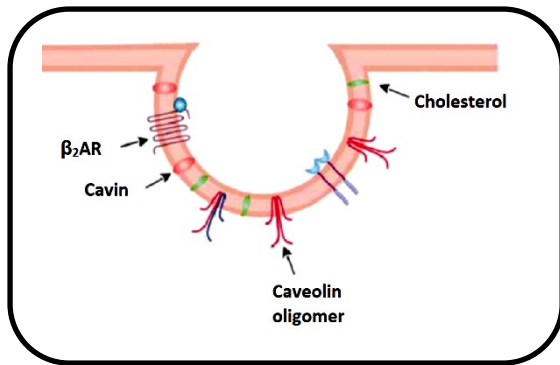


Figure 1. 5 β_2AR s are localised to membrane caveolae in healthy adult myocytes

Caveolae are specialised lipid rafts enriched with cholesterol, sphingolipids and a combination of caveolin proteins. They are known to house β_2AR , thus compartmentalising β_2AR -derived cAMP-dependent signalling and dampening the contractile response of the cells (Xu et al., 2015).

1.3.4 βAR association with caveolae

In cardiomyocytes, Cav3 have been known to directly interact with βAR s, G_s and G_i - α subunits, AC5 and 6 and also the regulatory subunit of PKAII (Prajapati et al., 2018). The interaction of these signalling proteins with the scaffolding proteins of caveolae also play a regulating their intrinsic activity (Snir et al., 2003). There has been evidence suggesting that cytoskeletal interactions play a role in targeting and stabilising caveolar proteins as disruptions to microtubules and actin filaments lead to a delocalisation of signalling proteins from the caveolar fractions (Shadrin et al., 2017). The localisation of some signalling proteins to different membrane domains can be affected by receptor activation which lead to dynamic changes in protein composition (Giacomelli et al., 2020). For example, β_2AR s are normally located in the caveolar membrane fractions. Upon agonist stimulation, the receptors become phosphorylated by G-protein coupled receptor kinases (GRK) which leads to the binding of the adapter protein β -arrestin triggering receptor internalisation (Ronaldson-Bouchard et al., 2019). It has been previously been shown that receptor internalisation is necessary to activate the G_i -signalling pathway (Mills et al., 2017). In addition, receptor internalisation also recruits binding of PDEs (Prajapati et al., 2018).

Although β_1 ARs signalling has far reaching effects, they are found in all fractions of the plasma membrane, caveolar and non-caveolar lipid raft fractions (Snir et al., 2003). Many of these studies have also highlighted that G-stimulatory proteins, which couple to β ARs and AC, display similar distribution patterns. Evidence suggests that localisation of AC5/6 to caveolar fractions could be due to direct interactions with Cav3 (Shadrin et al., 2017). It was uncertain whether cAMP produced by β_1 ARs and β_2 ARs localised variably throughout the membrane domains plays an equal role in regulating the various PKA targets or not. This was investigated by disrupting the caveolae microdomains by depleting the membrane cholesterol with the treatment of M β CD or by knocking down Cav3 expression with siRNA. It was shown that neither cholesterol depletion nor Cav3 knockdown effected beating rate upon maximal β_1 AR activation or LTCC function. There was also no change in the maximal contraction or LTCC response detected in adult ventricular myocytes upon β_1 AR stimulation. However, there are indications that at submaximal levels, cholesterol depletion can enhance sensitivity of cardiomyocytes to β_1 AR stimulation, and of contraction and LTCC without altering maximal response (Balijepalli et al., 2006). It has been proposed that this is because the loss of cholesterol from caveolae disrupts the inhibitory effect that Cav3 would have on AC when it interacts with it.

1.3.5 PDEs compartmentation

Several studies demonstrate that the inhibition of PDE activity effects intracellular cAMP gradients (Mills et al., 2017). Electrophysiological and imaging approaches have provided critical insight into the key role PDEs play in shaping intracellular gradients of cAMP (Prajapati et al., 2018). It is suggested that the mechanisms through which PDEs regulate cAMP activity and its diffusion is based on the localisation of PDEs to targeted subcellular compartments. This has been demonstrated previously by Mongillo et al where they studied the activity of PDE3 and PDE4 in neonatal cardiomyocytes upon β AR stimulation and found vast differences in their activity (Mongillo et al., 2006). They reported, that

although PDE3 contributes to 30% of all PDE activity, yet its complete inhibition resulted in only a marginal effect on cAMP. On the other hand, inhibition of only 10% of PDE4 activity resulted in a substantial increase in cAMP. Immunofluorescence staining revealed that PDE3 and PDE4 are localised to distinct cellular compartments of the adult myocyte, such as the microsomal fraction and the Z-band respectively (Vandeout et al., 2007; Richter et al., 2011). Similarly, PDE2 was shown to be localised to specific microdomains, and despite representing only about 1% of all PDE activity, it was responsible for degrading large amounts of β AR-produced cAMP (Giacomelli et al., 2020). This indicates that there may be functional coupling between localised PDEs and selected pools of AC, so specific receptor activation leads to a targeted response of cAMP and its associated PDE. Terrin et al further demonstrate that the specific arrangement of different PDEs results in spatially defined patterns of local cAMP pools and therefore the production of cAMP gradients throughout the myocyte (Terrin et al., 2006).

Localisation of PDEs to specific subcellular compartments is controlled by different mechanisms which facilitate the direct binding of the PDE to the cell membrane lipids or to particular proteins (Terrin et al., 2006). For example, previous literature suggests PDE4 isoforms are able to interact with AKAPs, such as PDE4D3 can bind to mAKAP in muscles and also to AKAP 450 at the centrosomes (Baillie et al., 2005) and so can regulate PKA activity. Upon β AR stimulation, cAMP levels in the cell rise and anchored PKA molecules become activated. This leads to the phosphorylation of PDE4D3, resulting in cAMP degradation, as well as enhancing PDE4D3's affinity to mAKAP. Therefore, as PDE4D3 is drawn towards mAKAP complexes with increased activity, cAMP levels return to basal levels (Dodge-Kafta et al., 2005, Baillie et al., 2005). This negative feedback loop of locally fluctuating cAMP and PKA activity generates the co-localisation between PKA and PDE, and has been confirmed by imaging studies in live cells.

1.4 INDUCED PLURIPOTENT STEM CELL DERIVED CARDIOMYOCYTES

1.4.1 Generation of iPSC-CMs from iPSCs

Stem cells are a class of undifferentiated cells that self-renew through mitotic cell divisions and have the ability to differentiate into specialised cell types (Hochdinger & Path, 2009; Sinnecker et al., 2012). Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) are both pluripotent and can differentiate into a range of somatic cell types (Hochdinger & Path, 2009). Human ESCs are derived from the inner cell mass of human blastocysts and were first successfully established by Thomas et al. (Thomas et al., 1998). hESCs can be differentiated into a range of cell types, including cardiomyocytes (Laflamme et al., 2007). However, the therapeutic use of hESCs is not risk free. Evidence of teratoma formation (Nussbaum et al., 2007) and immune rejection have been observed which, along with the ethical concerns of destroying human embryos, raises concerns about the use of hESCs which are yet to be resolved.

Subsequently, the reprogramming of somatic tissue to generate induced pluripotent stem cells (iPSCs) is one of the greatest developments in biomedical science and has since propelled the field of regenerative medicine. iPSCs provide an unlimited source of cells in a patient- and tissue-specific manner with reduced risk of host immune rejection. In addition, it allows pharmacological testing for patient-specific phenotypes and genotypes (Khan et al., 2013).

Over a decade ago by Yamanaka and colleagues succeeded in producing iPSC by the use of four transcription factors, OCT4, SOX2, KLF4, and c-MYC. iPSCs displayed very similar characteristics to ESC in their capacity to self-renew and the ability to differentiate into various cell types, and also showed genome-wide similarities (Yu et al., 2007). For differentiating hPSCs into cardiomyocytes, two basic approaches have been used; 1) embryoid body (EB) method (Kehat et al, 2001), and 2) 2D monolayer differentiation on extracellular matrix (Zhang et al., 2009). Initial studies using the EB method aimed to recapitulate the initial stages of embryogenesis. This method requires stem cells to be aggregated

by suspension, which leads to the EBs containing differentiated cell types from all three germ layers. These are then dissociated and plated on culture dishes. They were cultured for around 10 days, when spontaneous contracting developed in 5-15% of EBs (Kehat et al., 2001; Mumery et al., 2012; Zwi et al., 2009). However, this method of cardiomyocyte development was very inefficient and had low yields and poor cardiomyocyte quality due to the variability in size and density of EBs (Habib et al., 2008; Vidarsson et al., 2010). Furthermore, due to the use of serum in the protocol, additional heterogeneity was introduced, resulting in reduced efficiency (Xu et al. 2002). This has led to the development of monolayer-based 2D-culture methods.

1.4.2 Applications of iPSC-CMs

Myocardial infarction leads to a significant amount of cell loss, and with the heart having limited regenerative potential, the contractile cardiomyocytes are replaced with scar tissue which reduces myocardial function (Bergmann et al., 2009; Koudstaal et al., 2013). Currently, therapies for heart failure focus on preventing the progression of disease and to relieve symptoms, with cardiac transplantation being the only treatment option for end-stage heart failure (Gridelli & Remuzzi, 2000; Trulock et al., 2007). Thus, there is an urgent need for the development of regenerative treatments.

A range of cell types to serve as sources of new cardiomyocytes have been explored, including skeletal myoblasts, bone-marrow-derived mononuclear cells, mesenchymal stem cells and adult cardiac stem cells to name a few (Gnecchi et al., 2008). Although the use of these cells to repair myocardial tissue has been investigated in clinical trials, the efficacy with which they do so is marginal (Shintani et al., 2009). More recent studies are investigating paracrine factors that can trigger proliferation and differentiation of endogenous cardiac progenitors towards cardiomyocytes (Reinecke & Murry, 2000). Also, the direct reprogramming of resident cardiac fibroblasts into cardiomyocytes also poses a

potential therapeutic avenue as it would provide non-immunogenic, autologously derived cardiomyocytes (Ieda et al., 2010).

The use of iPSC-CMs also provides great possibilities for a range of other therapeutic applications and are invaluable tools for the investigation of development, disease processes and drug or toxicity screening. The ability to generate large numbers of human cardiomyocytes allows the acceleration of progress in numerous research areas. Some of the potential uses of iPSC-CMs are highlighted in Fig 1.6 below. Despite its many applications, the following sections will focus mainly on cell therapy, disease modelling and drug discovery studies.

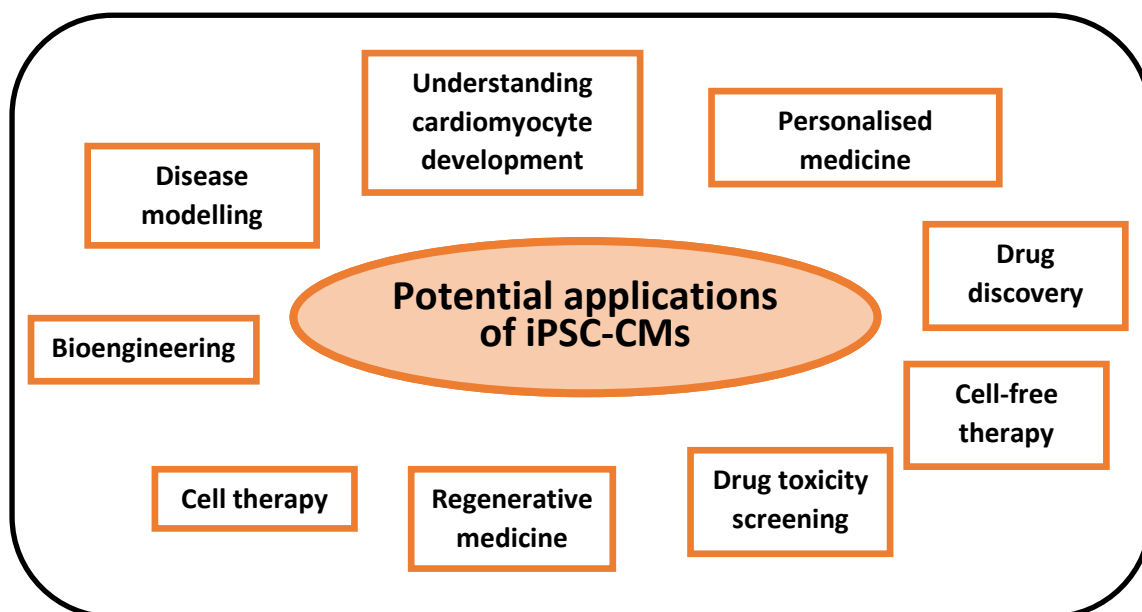


Figure 1. 6 Potential applications of iPSC-CMs

A basic overview of potential applications of human iPSC-derived cardiomyocytes for precision medicine. The differentiation of iPSCs into myocytes provides an effective platform for various disease modelling, cell-based or cell-free therapies, drug testing, bioengineered tissue construction among many other beneficial applications.

1.4.2.1 Cell therapy and cardiac regeneration

The use of iPSC technology to efficiently and reproducibly generate cardiomyocytes is becoming an attractive approach in cell therapy, particularly for the following reasons. Firstly, iPSCs have extensive differentiation potential, therefore allowing large quantities of cardiomyocytes to be generated. Secondly, they are self-renewing. This makes the induction of desired genetic modifications prior to differentiation more feasible if needed (Burridge et al., 2012). iPSC-CMs are known to exhibit contractile properties and have been shown to successfully engraft into the host myocardium with efficient electromechanical coupling to host cardiomyocytes (Shiba et al., 2016). Furthermore, iPSC-CMs can be derived from a patient's own somatic cells, thus avoiding potential immunogenicity of transplanted cells (de Almeida et al., 2013).

Many previous studies in animal models of cardiac disease have highlighted an improvement in cardiac physiology upon cell therapy. Despite these positive results, the initially noted functional improvement is usually seen to diminish with the progression of time (Alaiti et al., 2010; Reinecke & Murry, 2000). More recently, it is believed that myogenesis occurs over a longer period of time, however, the positive changes seen had occurred much earlier, therefore the detected improvements in cardiac physiology could not be a result of myogenesis (Alaiti et al., 2010; Grimaldi et al., 2013). Instead, it's due to paracrine signalling where the implanted cardiomyocytes release soluble mediators which are influencing cardiac response. However, there are further discrepancies in current literature regarding the effect and mechanism behind paracrine signalling in the myocardium (Snir et al., 2003a; Veerman et al., 2015; Zhang et al., 2009), and Reinecke et al point out the low retention and substantial death of grafted cells, suggesting that these improvements in myocardial function cannot be due to myogenesis alone. Therefore, other mechanisms need to be investigated (Reinecke et al., 2002; Snir et al., 2003a; Veerman et al., 2015; Zhang et al., 2009).

Several other concerns with the use of iPSC-CMs for cell therapy have also risen, such as cell source, dose, timing and delivery methods which also need to be addressed. In addition, cell engraftment and

cell survival remain major problems, particularly for the treatment of large damaged areas. It is also important to study the 3D reorganisation of cells when engrafted to ensure correct orientation for effective contraction. Despite the major progress made in understanding iPSC-CMs and their functional contribution to the damaged heart, there are many critical concerns which need to be addressed for effective cell therapy in restoring cardiovascular function (Lundy et al., 2013a; Snir et al., 2003a). A major concern is the level of cellular maturity of iPSC-CMs. They display limited ultrastructural, electrophysiological, and metabolic features, and is described further in section 1.5 below.

1.4.2.2 Disease modelling

hiPSC lines have been developed from many individuals with various cardiac diseases, particularly with ion channel gene or sarcomeric protein mutations (Brandao et al., 2017; Giacomelli et al., 2017). Ion channel mutations can lead to arrhythmias and occasionally sudden cardiac death. The use of hiPSC-CMs in recapitulating the disease phenotypes have also allowed us to identify underlying disease mechanisms as well as indicating treatment modalities. Studying ion channelopathies are of particular interest as ion channels of smaller hearts, of rodents for example, are functionally different to human, so relevant animal models are unavailable. Since ion channels are expressed within 2 weeks of cardiomyocyte differentiation in hiPSC-CMs, cell immaturity has little impact on predictive outcomes for adult humans, making iPSC-CMs a good model to study channelopathies. In addition, numerous other diseases have been investigated *in vitro* through the use of patient-derived iPSC-CMs, such as Leopard syndrome, Timothy syndrome, Long QT syndrome, familial hypertrophic and dilated cardiomyopathy (Carvajal-Vergara et al., 2010; Lan et al., 2013; Moretti et al., 2010; Sun et al., 2012; Yazawa et al., 2011). Despite their great potential in elucidating the underlying disease mechanism and developing novel therapeutics, iPSC-CMs are still not a true representation of the *in vivo* myocardium and some diseases require greater maturation stages of cardiomyocyte development.

1.4.2.3 Screening for drug discovery

Human iPSC-CMs provide the ability to study cellular signalling, metabolism and a range of ion channel functions in cardiomyocytes. Therefore, many hiPSC-CM disease models allow the development of novel drugs or the discovery of potentially new therapeutic targets with the aim to improve cardiac function. Estimates suggest that it costs roughly \$1.24 billion to bring a new drug to the market, including the cost of failures (Kaitin, 2010). Many drugs never make it to market or are withdrawn after initial approval due to unforeseen cardiotoxicity as clinical trials relied on the use of non-human cells, such as Chinese hamster ovary cells, or transgenically modified human cells lines, such as human embryonic kidney cells. Human iPSCs can be used to for pharmacological testing for toxicity on almost any human tissue or cell type. In addition, major differences between human and animal physiology, such as drug metabolism or mechanism of action can be limited.

1.5 COMPARISON OF iPSC AND ADULT CARDIOMYOCYTES

Despite vast developments in producing large numbers of iPSC-CMs efficiently, there are still some problems which remain to be addressed. Over the years, we have significantly developed our differentiation protocols, however the iPSC-CMs generated remain qualitatively and quantitatively immature (Goversen et al., 2018; Lundy et al., 2013; Snir et al., 2003). Previous literature show hiPSC-CMs display underdeveloped ultrastructural, electrophysiological, and metabolic features. In some cases, these differences between hiPSC-CMs and adult cardiomyocytes can lead to inaccurate disease modelling or drug testing, making clinical translation difficult. For example, when studying the effect of drugs on contractile function, the need for fully developed contractile characteristics is vital, and immature iPSC-CMs may prove an inaccurate model.

1.5.1 A comparison of structural properties of adult- and iPSC- cardiomyocytes

The size and shape of iPSC-CMs is influenced by the environment in which they are cultured. Generally, flat, round or irregular shapes and disorganised internal structure are observed (Lundy et al., 2013; Mummery et al., 2003; Snir et al., 2003; Zhang et al., 2009), resembling more of a foetal-like cardiomyocyte. There are significant structural developments cardiomyocytes undergo between the neonatal to adult transition. To begin with, adult cardiomyocytes possess a high length-to-width ratio, developing an elongated, rod-like shape. This allows for efficient electrical conduction and muscle contraction (Bird et al., 2003; Peters et al., 1993). However, iPSC-CMs in culture do not undergo physiological hypertrophy for increasing cell size in the same manner as human cardiomyocytes do *in vivo*, therefore they remain much smaller than adult cardiomyocytes (Maillet et al., 2013; Smolich, 1995).

Furthermore, adult cardiomyocytes display highly organised functional sarcomeric units: Z-disks, I-, H-, A- and M-bands (Fig 1.1) (Gregorio & Antin, 2000). In addition, adult cardiomyocytes have sarcomeres about 2.2µm in length (Denning et al., 2016). Sarcomeres consist of thick and thin filaments of proteins and are essential for the formation of contractile units, determining the force of cell contraction (Hwang & Sykes, 2015). At the initial stages of development, foetal cardiomyocytes, have sparsely distributed myofilaments in no regular orientation. The myofibrils sarcomeres begin to align, with the thick and thin filaments becoming denser, though full maturity is not yet reached (Kim et al., 1992). In contrast, iPSC-CMs display irregular morphology, and have sparsely distributed myofilaments in no regular orientation, resembling the ultrastructural features of underdeveloped cardiomyocytes, such as disorganised sarcomeres which are relatively short (1.6 µm) in length (Bird et al., 2003; Feric & Radisic, 2016; Kim et al., 1992). In addition, developing cardiomyocytes are generally 30-fold small than adult cardiomyocytes, thus do not generate strong contractile force in the same way as adult myocytes, leading to different electrical signalling characteristics (Scuderi & Butcher, 2017).

Due to nuclear division without cell division, approximately 25-30% of adult cells are bi-nuclear. Late foetal, early postnatal myocytes and iPSC-CMs are usually mononucleated, and occasionally binucleated with the nucleus occupying around 10-20% of the cell volume (Snir et al., 2003; Smolich et al., 1995).

Mitochondria are the powerhouse of the cells, and their morphology and functional characteristics are also considered markers for myocyte maturation (Dai et al., 2017; Yang et al., 2014). Adult cardiomyocytes tend to possess a large number of mitochondria which are evenly distributed and account for 20-40% of the cell size (Denning et al., 2016a; Kim et al., 1992; Olivetti et al., 1996). Mitochondria in early cardiomyocytes tend to be smaller and rounder in shape dispersed throughout the cell cytoplasm. Throughout development, they become cylindrical in shape and occupy 35% of the cell volume (Kim et al., 1992; Smolich, 1995). The mitochondria are aligned along the sarcomeres and become localised towards the pole of the nuclei, many are also found in the sub-sarcolemmal space.

This is essential for myocytes in order to support the energy requirements during contraction. Like foetal cardiomyocytes, iPSC-CMs have longer, elongated mitochondria which are diffused throughout the cell and found to cluster to the periphery (Gherghiceanu et al., 2011). Adult cardiomyocytes rely on fatty acid oxidation as the source of energy production. The switch from glycolysis to fatty acid oxidation occurs as the foetal heart matures. However, iPSC-CMs rely on glycolysis as their main source of energy (Lopaschuk & Jaswal, 2010).

Along with sarcomeric components, adult cardiomyocytes form other highly sophisticated internal structures, such as T-tubules (Fig 1.7). These are membrane invaginations present at regular intervals along the Z-lines of adult cardiomyocytes. They are essential components for regulating contraction as they are involved in ECC for rapid electrical excitation, initiation and triggering of Ca^{2+} release from the SR (Ziman et al., 2010). As ventricular myocytes are large cells, the presence of T-tubules is essential for myocyte function. The disruption of T-tubules has been implicated in animal models of heart failure (Balijepalli et al., 2003). T-tubules are absent in early development and start to develop around 32 weeks in foetal cardiomyocytes (Kim et al., 1992). Literature shows that in ventricular cardiomyocytes, the development of these structures begins with the proliferation and merging of caveolae, these then extend through the cell's interior and couples with the SR (LeGrice et al., 1995). Multiple studies show that iPSC-CMs lack T-tubules. The absence leads to unsynchronised Ca^{2+} transients due to inhomogeneous Ca^{2+} release (Severs et al., 2008). Some studies have identified mechanisms of enhancing T-tubule development through 3D culture (Shardin et al., 2017), tri-cultures tissue incorporation (Giacomelli et al., 2020), and electromechanical manipulation (Ronald-Bouchard et al., 2019). Maturation techniques are discussed further in section 1.6.

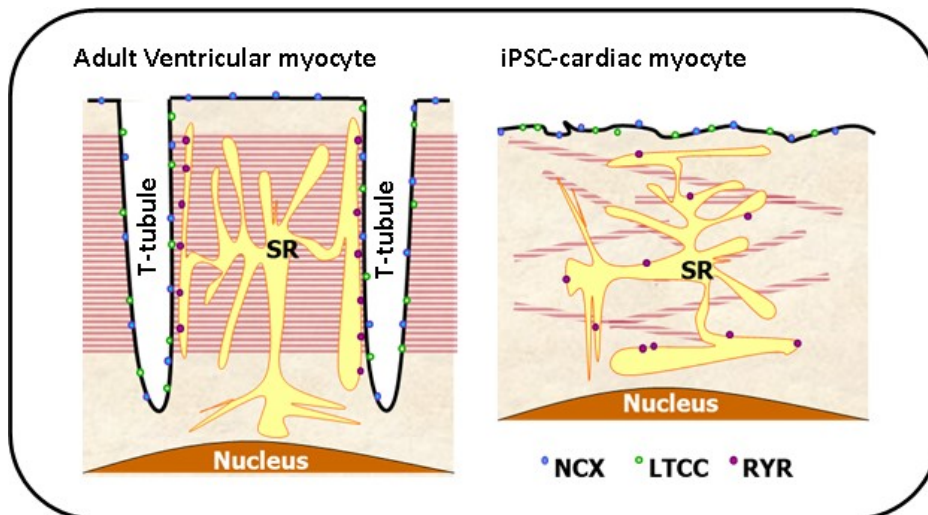


Figure 1. 7 Ultrastructural differences between adult ventricular myocytes and iPSC-CMs.

The lack of myofilament alignment and regularity, absence of T-tubules and disorganised sarcoplasmic reticulum is depicted in iPSC-CMs, resulting in irregular receptor and channel localisation. Adapted from: (Kane et al., 2015)

Intercalated discs are highly organised intracellular adhesion structures of the myocardium which connect adjacent cardiomyocytes in order for them to function as a single syncytium, thus supporting synchronized contraction of the cardiac tissue. They are composed of three specialised cell junctions: Fascia adherens (adheren junctions), macula adherens (desmosomes) and gap junctions (Sheikh et al, 2009). Fascia adherens and macula adherens are required for mechanically coupling and reinforcing a physical connection of neighbouring cardiomyocytes. The fascia adherens consist of a dense filamentous network to which thin filaments from the terminal ends of myofibrils connect. They are the primary anchoring sites to the actin cytoskeleton (Henderson et al., 2017). Desmosomes are smaller, denser modifications of the sarcolemma and are essential for intercellular adhesions. They are involved with force transmission between cardiomyocytes and play an important role in resisting against shearing forces (Lehnert et al., 2004; Henderson et al., 2017). Gap junctions are essential for electrical coupling between cardiomyocytes and allows rapid electrical transmission between cells (Peters et al., 1994; Severs et al., 2008). They consist of six connexin molecules which form two half-

channels into the intracellular space. Once they are connected to the connexin molecules of the adjacent myocytes, they form functional gap junctions and allow the passage of ions and small molecules between cells (Melax et al., 1969; Peters et al., 1994). In developing foetal cardiomyocytes, the intercalated discs are oblique and not well aligned. They display limited area of close apposition of neighbouring myocyte membranes. There is a gradual increase in the extent of intercalated discs during the pre-natal period with the adult cell appearance being established soon after birth (Lieu et al., 2009). iPSC-CMs however, are connected via primitive intercalated discs (Smolich, 1995). They display gap junctional proteins around the whole circumference of the cell with no specific localisation to the short axis. This results in an isotropic signal so conduction velocity and efficiency of signal propagation is reduced, thus contributing to the immature electrical response of the cell (Bers, 2002).

1.5.2 A comparison of functional properties of adult- and iPSC- cardiomyocytes

The cardiac action potential (AP) determines the changes in transmembrane electrical activity of the cell which occur during each cardiac cycle. An AP arises from the time- and voltage-dependant changes in activity of ion transporters. APs can vary greatly between various cardiomyocyte subtypes (i.e. atrial, ventricular or nodal cells), a feature known as electrical heterogeneity. Cardiomyocytes have a selectively permeable membrane with a combination of ion channels, exchangers and pumps that allow the cells to stably maintain a negative charge inside the cell. When the electrical and chemical forces generated by the intracellular and extracellular are balanced and there is no net movement of ions, and equilibrium potential is reached; known as the resting membrane potential. There is net movement of K ions at the resting membrane potential (I_{K1}) (Amin et al., 2010; Bers, 2002; Liu et al., 2016). The cardiomyocyte remains inactive until an action potential is initiated by a flow of ions through transmembrane ion channels from adjacent cells. In atrial and ventricular myocytes, the AP is divided into 5 phases (0-4): phase 0 is the depolarisation phase, phases 1-3 are the repolarisation

and plateau phases, and phase 4 is the resting phase) (Fig 1.8) as described earlier. There are notable differences between the action potential of adult cardiomyocytes and iPSC-CMs, shown below.

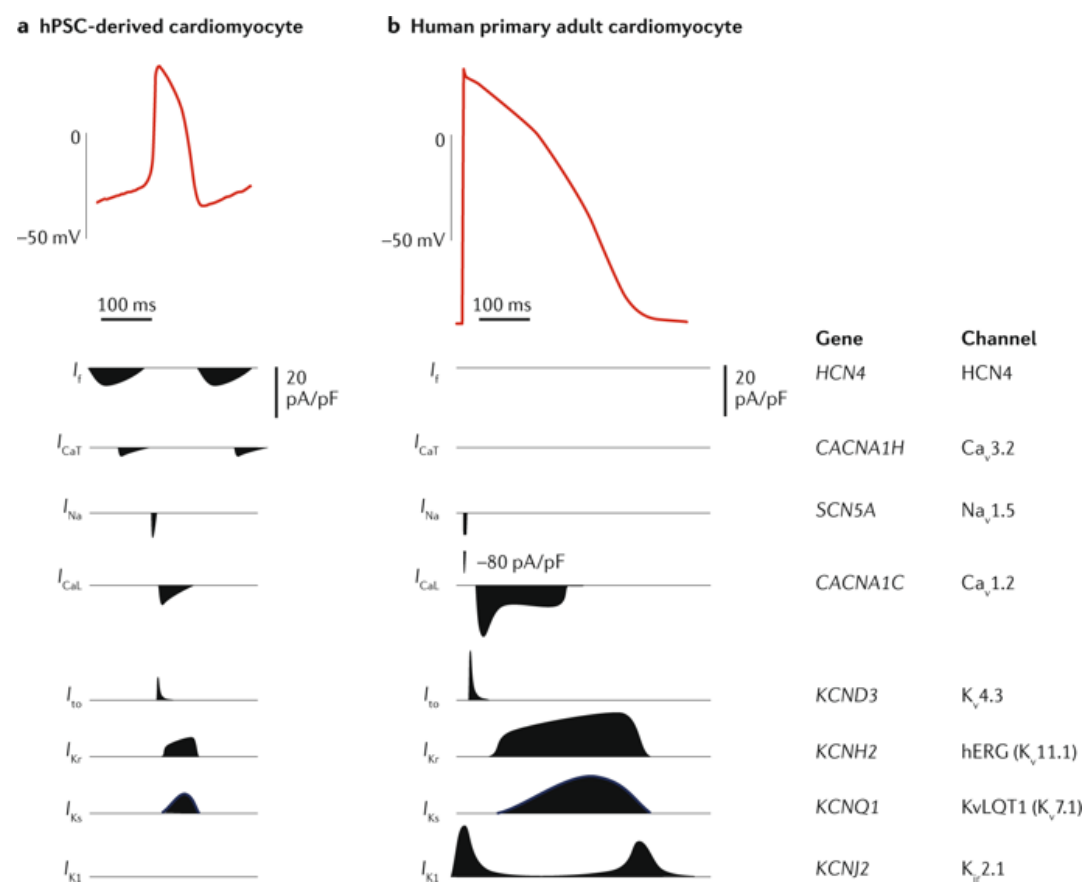


Figure 1. 8 Action potential recordings from iPSC-CMs and adult myocytes
Comparison of action potential recordings from a) iPSC-myocytes and b) isolated human primary left ventricular myocytes from phase 0 to 4 and the ion channels and genes with respective ion channel trajectories (Karbassi et al., 2020).

Mature adult myocytes only beat when they are stimulated with a force around 40-80 mN/mm², conduction velocity around 60 cm/s and an upstroke velocity of 150-350 V/s. However, iPSC-CMs these properties are 0.08-4 mN/mm², 10-20 cm/s, and 10-50 V/s respectively (Denning et al., 2016). Furthermore, iPSC-CMs can be of atrial, nodal or ventricular-like phenotype, therefore display mixed action potential morphologies (Ma et al., 2011). iPSC-CMs are known to exhibit important sodium,

potassium and calcium currents, however, they do not display inward rectifier current (IK_1) and so do not display stable a resting membrane potential (Dhamoon & Jalife, 2005; Hoekstra et al., 2012; Knollmann, 2013). In addition, iPSC-CMs display spontaneous beating. It has been suggested that high levels funny current (I_f) generated by hyperpolarization-activated cyclic nucleotide-gated (HCN) is responsible for the maintenance of this automaticity (Yanagi et al., 2007). Development from foetal to adult myocytes the number of cells expression IK_1 increase (Huynh et al., 1992). (I_f) in foetal cells is high at early stages, but through the maturation process HCN expression is down regulated. This decreases the funny current in ventricular myocytes as the adult stage of development is reached, however, this process fails to occur in iPSC-CMs and so they display automaticity (Zorn-Pauly et al., 2003). Therefore, developing an understanding of ion channels regulation can allow the generation of biological pacemakers was required and eliminating this property in myocytes. Assessing electrophysiological parameters during iPSC-CMs development is an important method of assessing maturity, although it is technically challenging.

Mature adult myocytes display well-developed and highly structured T-tubules and sarcoplasmic reticulum. These allow regulating of calcium-induced-calcium-release (CICR) and fast excitation contraction coupling (ECC). Calcium flows into the cell via the LTCCs, which triggers the release of calcium from the sarcoplasmic reticulum through the ryanodine receptors (Bers, 2002). During relaxation, calcium returns back to the sarco/endoplasmic reticulum calcium ATPase (SERCA) and exits the cell through the Na^+-Ca^{2+} exchanger (NCX). The uniformity of this intricate process is essential for the efficient, synchronized contraction of neighbouring sarcomeres (Scuderi & Butcher, 2017b; Steinhoff et al., 2017). In comparison, iPSC-CMs do not have T-tubules and display significantly underdeveloped sarcoplasmic reticulum as they do not possess the required regulatory proteins such as calsequestrin or phospholamban (Binah et al., 2007; Dolnikov et al., 2006; Liu et al., 2007). They also have low expression on SERCA and other key proteins such as calsequestrin, RyR2 and phospholamban (Dolnikov et al., 2006; Itzhaki et al., 2011; Liu et al., 2007; Rao et al., 2013; Synnergren

et al., 2012). Although foetal myocytes have similar expression levels of SERCA to adult myocytes, there is a greater level of posttranslational modifications of the SERCA2a mRNA as the cells mature (Qu & Boutjdir, 2001). iPSC-CMs display unsynchronised contraction, slow ECC and rely on LTCCs for the increase of calcium rather than through the sarcoplasmic reticulum, which prolongs the transient rise time (Pesi et al., 2017; Veerman et al., 2017).

The β AR signalling pathway is essential for mediating inotropic and chronotropic function for meeting the increased demand during cardiac stress (Rockman et al., 2002; Xiang & Kobilka, 2003). Adult myocytes co-express β_1 AR and β_2 AR at the ratio of 70-80%/30-20% in human ventricles (Brodde, 1991; del Monte et al., 1993; Engelhardt et al., 1996). Some literature suggests that the activation of both receptors leads to phosphorylation of phospholamban and cardiac troponin I, however, enhanced contractile function is predominantly conducted via the β_1 AR (del Monte et al., 1993; Kaumann et al., 1999). With consideration of the previously discussed immaturities of iPSC-CMs, it is understood that iPSC-CMs will express and respond differently to pharmacological adrenergic stimulation. A study conducted by Wu and colleagues further elaborated on β AR signalling pathway development in iPSC-CMs upon receptor stimulation. They show that iPSC-CMs express both β_1 ARs and β_2 ARs, however, β_1 AR displays a late onset expression pattern compared to β_2 AR (Wu et al., 2015). This differential regulation of β_1 AR and β_2 AR signalling is consistent with rodent studies, where β_1 AR stimulation leads to cAMP-mediated contraction in neonatal myocytes, whereas β_1 AR activity is more prominent in healthy adult myocytes (Slotkin et al., 1994).

Due to the expression pattern of these receptors, the adrenergic regulation of calcium handling and contraction force was initially dominated by the β_2 ARs, which then switched to β_1 AR with in vitro cell development and the increase in receptor expression (Wu et al., 2015). β_1 AR expression is shown to be dominant in cardiac progenitor cells also, and upon cardiac commitment leads to increased β_1 AR expression, indicating a similar pattern as detected in iPSC derived myocytes (Khan et al., 2013).

In adult ventricular myocytes, β AR stimulation enhances systolic and diastolic function by PKA-mediated activation of downstream effectors (Kaumann et al., 1999). It has been shown that β AR stimulation in iPSC-CMs results in increased chronotropic and inotropic responses. iPSC-CMs, unlike adult myocytes, do not display β_2 AR compartmentation and so its activation leads to a much greater contractile function. As iPSC-CMs develop, G_s expression levels are decreased and coupling with β_2 AR is also reduced. This led to a decreased regulatory role of β_2 AR as iPSC-CMs develop (Wu et al., 2015).

Another study conducted by Pillekamp and colleagues investigated the β AR and calcium handling properties of iPSC-CMs. They showed that when ESC-CMs were co-cultured with non-contractile neonatal mice ventricular slices, the removal of calcium or blocking the calcium channel stopped the spontaneous beating, whereas increasing intracellular calcium concentrations accelerated beating rate and isometric force. They also reported that β_1 AR stimulation resulted in a dose-dependent increase in myocyte beating frequency without effecting isometric force (Pillekamp et al., 2012). However, some studies report contradictory results. They detected an inotropic response to norepinephrine in both iPSC-CMs and ESC-CMs (Brito-Martins et al., 2008; Germanguz et al., 2011; Liu et al., 2012; Yokoo et al., 2009). In the foetal heart, expression of the β ARs is relatively low and various components of the β AR downstream signalling pathway are absent. This may explain the diminished physiological function and reduced or uncoordinated response in early myocytes (Slotkin et al., 1994).

Another key difference observed is the lack of compartmentation of the β_2 ARs within caveolae or T-tubules in iPSC-CMs. In adult myocytes, β_2 ARs are restricted to specialised microdomains of the cell membrane which makes them unable to phosphorylate certain downstream mediators (Nikolaev et al., 2006). The absence of T-tubules and caveolae in iPSC-CMs leads to persistent coupling of β_2 ARs to downstream effectors resulting in immature signalling.

In summary, the use of iPSC-CMs is a potentially viable alternative for the repair of the damaged myocardium. However, there are several hurdles which need to be overcome for impactful translation in the clinical setting. Despite various methods of iPSC differentiation into myocytes have been investigated, iPSC-CMs are generally considered immature as they do not display the sub-cellular, cellular and tissue-level organisation in the same way as adult myocytes. Therefore, the maturation of iPSC-CMs is indispensable in order to fulfil the physiological functions required by the cell, such as calcium handling, intracellular signalling and contractile properties. Changes of these properties in health and disease are further summarised in figure 1.9.

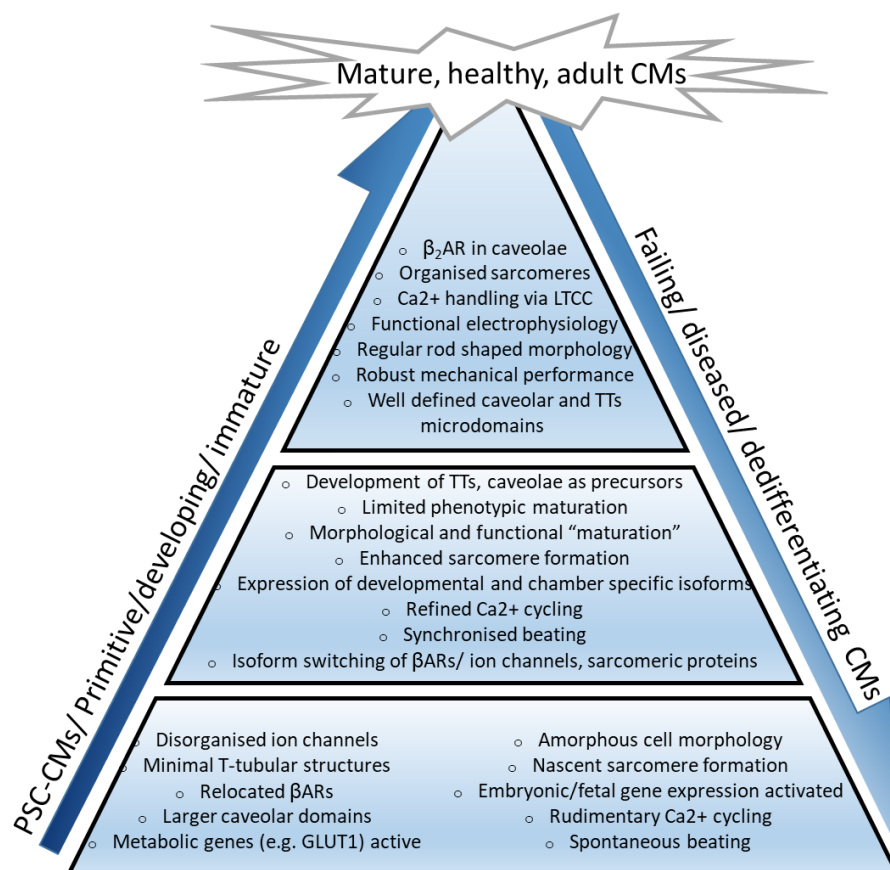


Figure 1. 9 Summary of the developmental progression of cardiomyocytes

The pyramid depicts the transition from primitive, underdeveloped myocytes to more mature, well-developed myocytes and the recurrence of this developmental transition at the onset of disease.

1.6 METHODS OF MATURATION OF CARDIOMYOCYTES

In order to overcome the cellular immaturity, many studies are investigating physical, chemical, genetic and environmental methods of maturation in vitro to obtain a cellular phenotype which is representative of the human adult myocardium (Fig1.10). It has been observed through various independent studies that myocyte morphology, sarcomeric protein content and organisation are crucial for maturation. To achieve this, numerous methods have been employed. For example, biophysical stimuli such as mechanical cues, topography, electrical stimulation and using optimal substrate stiffness is important. In addition, the use of biochemical cues such as the use of soluble substrate ligands is also thought to influence maturity. Numerous other techniques such as genetic manipulation, modified culture conditions and physical and electrical stimuli have also been investigated. Many existing reviews summaries these methods in greater detail (Denning et al., 2016; Veerman et al., 2015; Yang, et al., 2014), with a few approaches being discusses below.

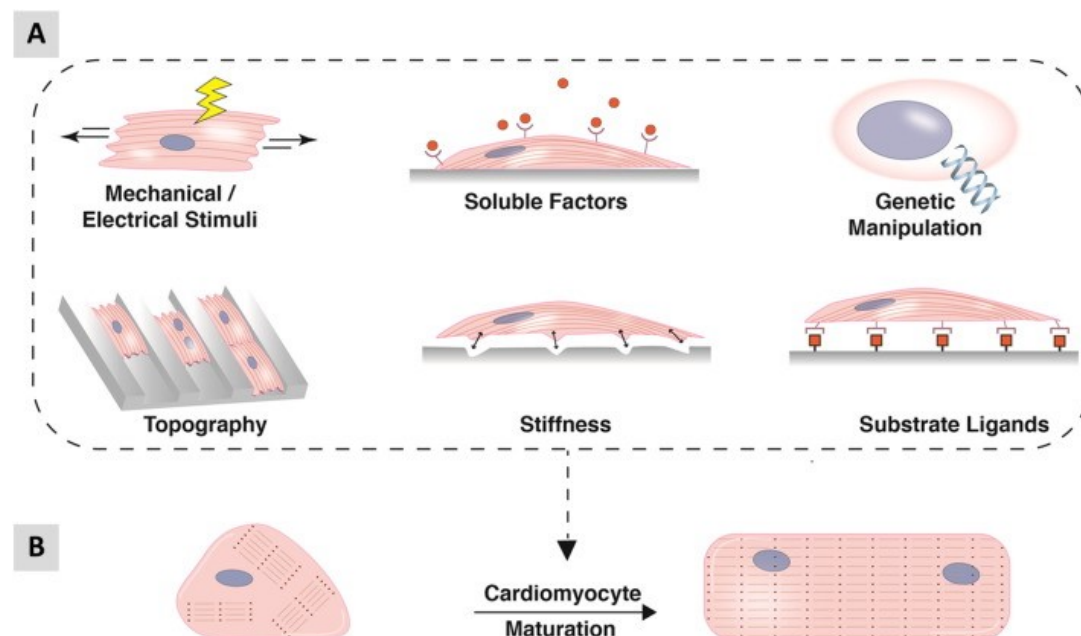


Figure 1. 10 Mechanisms of iPSC-CMs maturation

Process of iPSC-derived cardiomyocyte maturation in vitro, including biophysical, biochemical and genetic manipulation strategies with the aim to induce improved morphology, organised myofibrils alignment as well as functional properties for clinical application and disease modelling. Currently, iPSC differentiation can lead to myocytes with variable phenotypes and cellular characteristics with heterogenous levels of maturity. Differentiation can also lead to a non-cardiomyocyte population, such as endothelial and vascular smooth muscle cells. Therefore, a more specific methods for cellular differentiation and maturation are needed (Denning et al., 2016).

1.6.1 iPSC-CMs maturation by long term culture

Myocyte maturation is a gradual process and obtaining the developed adult phenotype *in vivo* can take up to 10 years for human neonatal cardiomyocytes (Peters et al., 2014). However, *in vitro* functional hPSC-CMs can be generated within 15 days of differentiation (Lian et al. 2012). As described above, the structural phenotype is fundamental for mature cardiomyocytes and maturation of these properties can significantly improve the potential for clinical applications. Many promising approaches to produce more mature iPSC-CMs have been investigated and many indicate that the process of maturation can be driven by mimicking *in vivo* microenvironments (Laflamme et al., 2011).

As described above, adult cardiomyocytes are large and cylindrical in shape, with dimension of 150 x 10µm of ventricular myocytes (Lundy et al., 2013). Embryonic and fetal myocytes are much smaller with variable dimensions (Kamakura et al., 2013). One study assessed how the length-to-width ratio develops as iPSC-CMs are matured with prolonged time in culture. They observed that early iPSC-CMs (initiation of contraction, <21 days) have a diameter of 5-10µm (Lewandowski et al., 2018) and late iPSC-CMs (>35 days) develop a more oblong morphology with dimension around 30 x 10µm (Lundy et al., 2013); although this is closer to an embryonic myocyte, it still remains smaller than adult myocytes.

Snir et al report that after 50 days of culture, hPSC-CMs undergo structural maturation and increase in size and length, although they remain small than adult cardiomyocytes. Furthermore, they showed that over this time, ESC-CMs begin to withdraw from the cell cycle, and also develop their cell length

to width ratio, along with improved sarcomeric organisation (Cui et al., 2007; Snir et al., 2003). Another study conducted by Lundy et al. showed that culturing hPSC-CMs up to 100 days significantly improves morphological characteristics of the cell, such as multinucleation, an increase in sarcomere length, cell size and an elongated shape (Cowan et al., 2004). Furthermore, a study investigating similar maturation aspects showed that culturing hPSC-CMs for 30 days improves Z- and I-bands, up to 90 days of culture improves A- bands, and one year of culture led to well-developed M-bands (McDevitt et al., 2005; Snir et al., 2003). In addition, Lewandowski et al also report that prolonged 40-day culture results in cellular hypertrophy with expression of genetic markers to similar levels to human myocardial ventricular cells; such as increased *CX43* expression, MHC isoform switch and troponin I isoform transition (Lewandowski et al., 2017). Other independent studies observed an increase in multinucleation and upregulation of MYH7, MYH6, GJA1 and SERCA2 gene expression (Norström et al., 2006; Porrello et al., 2011).

The proliferative activity of adult human cardiomyocytes is almost nil (Bergmann et al., 2009). Early iPSC-CMs as well as foetal and embryonic cardiomyocytes can display proliferative potential (Brito-Martins et al., 2008; Pillekamp et al., 2012). However, with prolonged culture time, the proliferative capacity of iPSC-CMs had lowered from 24-48h doubling time, to only 10% of cells being BrdU+ (bromodeoxyuridine, used to detect proliferating cells) at 4 weeks (Brito-Martins et al., 2008; Germanguz et al., 2011). This indicates that the proliferation rate of iPSC-CMs decreases as they age, with late cells losing proliferative power.

Long term culture has also shown to affect several key inotropic and chronotropic responses in iPSC-CMs. The β AR responses have been previously demonstrated in iPSC-CMs (Kehat et al., 2001; Xu et al., 2002) by stimulation of the β_1 ARs and β_2 ARs with isoprenaline resulting in a positive response (Kehat et al., 2001; Norström et al., 2006) regardless of cell line origin or differentiation method. However, unlike adult myocytes, along with increased contraction rate, increased calcium transient amplitude and decreased relaxation time, iPSC-CMs do not display an increase in contraction force

(Burridge et al., 2011). Similar to foetal cardiomyocytes, the β_2 AR response in iPSC-CMs and ESC-CMs accounts for 17-37% of the total response when simulated with isoprenaline (Brito-Martins et al., 2008; Zwi et al., 2009). Evidence suggests, with increased time in culture, iPSC-CMs display an amplified chronotropic β AR agonist response (Reppel et al., 2004; Zhang et al., 2009). In addition, when the phosphodiesterase inhibitor was applied to iPSC-CMs, an increase in intracellular cAMP and an increase in contraction rate was detected (Zhang et al., 2009), similarly with the application of the adenylyl cyclase activator forskolin (Bray et al., 2008a; Guven et al., 2015; Radisic et al., 2003). However, to date, it is unclear how maturation time influences the magnitude of these responses.

Time in culture has also shown to affect myocyte beating rate. At the initial stages of iPSC-CM differentiation, spontaneous and synchronous contraction within the monolayer can be seen from day 5 (McDevitt et al., 2002). Typically, the basal rhythm has been reported to range from 21 to 52 beats per minute (Bursac et al., 2002; Camelliti et al., 2006), with the majority reporting around 40 beats per minute (Ribeiro et al., 2015). The rate of contraction is thought to be due to differences in cell lines, culture conditions, time since differentiation or the onset of contraction. Various studies have reported slight increases in contraction rate; ranging from 40-85 beats per minute at 60 days (Chopra et al., 2011; Engler et al., 2008).

In summary, these studies suggest that prolonging culture time allows iPSC-CMs to naturally mature both structurally and functionally. However, these reports indicate that an adult cardiomyocyte phenotype is not completely achieved and suggests an additional method of manipulation is required.

1.6.2 iPSC-CMs maturation by micropatterning

In the native myocardial tissue, healthy adult cardiomyocytes have a very specific and organised structural pattern. They are elongated, rod-shaped cells with a length-to-width ratio of 7:1 and are

organised into parallel cardiac muscle fibres with intracellular contractile myofibrils, aligned along the axis of stretch to produce an isotropic tissue sheet (Kaji, et al., 2003). This highly specified cytoarchitecture allows electromechanical coupling of myocytes for directed contraction of the myocardium. This shape and alignment are due to external stimuli, such as topographical cues and signals from the extracellular matrix, to which the cardiomyocyte is exposed. Therefore, developing potential mechanisms for mimicking the *in vivo* myocardial environment may allow the production of a more mature phenotype in iPSC-CMs.

Previous studies have adopted various methods for producing aligned myocyte cultures. A study conducted by McDevitt *et al* (2002) microcontact printed lanes of laminin ranging from 5µm to 50µm in width on a non-adhesive surface. Neonatal rat ventricular myocytes adhered to the laminin lanes and adopted an elongated, rod-shaped pattern. The patterned cells displayed bipolar localisation of N-cadherins and connexin43 molecules, resembling intercalated disks (McDevitt et al., 2002; Feinberg et al., 2012). This is in accordance with other studies demonstrating improved structural and functional characteristics of neonatal myocytes after micropatterning (Ribeiro et al., 2015).

Another study explored the idea of micropatterning extracellular matrix components, such as fibronectin or Matrigel, to assess the effect of cell shape and substrate stiffness on shortening and movement of sarcomeres and its translation to mechanical output in live iPSC-CMs (Kim et al., 2010). When single iPSC-CMs were cultured on polyacrylamide substrates with defined aspect ratio, it was shown that cells adopted the shape of the micropattern (Fig 1.11). In addition, they detected improved contractile activity, calcium flow, mitochondrial organisation and electrophysiology. Other studies have also reported the positive influence of culturing cardiomyocytes on substrates with a physiological stiffness on myofibril organisation and cell function (Wang et al., 2011). Similarly, Kaji et al show that patterned myocytes are able to conjugate with each other, thus successfully forming gap junctions for calcium propagation, which led to synchronised contraction (Kaji et al., 2003). Further to this study and many others, the effect of enhance calcium handling by improve alignment of the

cardiomyocytes has also been investigated. There is evidence to suggest that increasing sarcomeric alignment increases force generation (Duval et al., 2017; Mirbagheri et al., 2019).

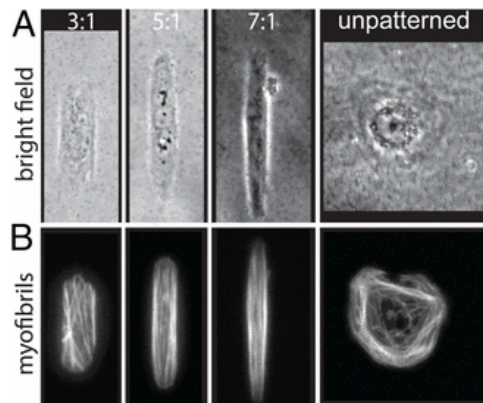


Figure 1. 11 Using micropatterns for structural maturation of iPSC-CMs

Matrix micropatterns help constrain hPSC-CMs to specific cellular dimensions and can engineer their mechanical output. Cells cultured on micropatterns possess desired aspect ratios for 1:1 to 7:1. Actin staining of myofibrils in micropatterned iPSC-CMs have greater alignment than unpatterned cells (Ribeiro et al., 2015).

Alternative to chemical patterning, the use of materials with ridges or nanogrooves for regulating myocyte morphology and alignment have also been investigated. A polyethylene glycol (PEG) construct with grooves of 150-800nm in width and 100nm in depth were produced. When NRVMs were cultured on these substrates, they aligned along the grooves and displayed anisotropic action potential propagation, contractility and conduction velocity similar to the native tissue, and increased expression of cell-cell coupling proteins (Figtree et al., 2017; Kelm et al., 2004). Furthermore, a study utilising nanogrooves of 450nm in width and 100-350nm highlighted that the nanogroove structures influence morphology and orientation of the attached myocytes, whereas the surface topography and the substrate stiffness regulated the organisation of the actin cytoskeleton and focal adhesion complexes, thus, were responsible for the contractile function of the cells (Correia et al., 2018; Huethorst et al., 2016; Lemoine et al., 2017; Ulmer et al., 2018). Shortly after, Rao et al also adopted topographical patterning by seeding iPSC-CMs on fibronectin coated PDMS which had microgrooves

of 10µm in width and 4µm in depth. They showed that after two weeks in culture, iPSC-CMs had improved cellular alignment and organised sarcomeres. Furthermore, patterned iPSC-CMs had improved calcium-handling properties, with a shorter time to peak and more organised calcium release (Tulloch et al., 2011).

1.6.3 iPSC-CMs maturation by 3D cultures

The heart is a complex organ composed of different cell types embedded in extracellular matrix proteins and are exposed to a vast range of biochemical, mechanical, electrical stimuli among others. This allows myocyte responses to be fine-tuned and appropriate to the stimulus. The use of multi-well plastic culture dishes is effective for expanding cell lines, cell differentiation and cell culture at defined conditions. However, 2D cultures are unable to recapitulate the complexity of the *in vivo* myocardial condition such as cell cross-talk, tissue architecture and the extracellular micro environments, therefore their signalling mechanisms and response to drugs and toxins can be inaccurate. Therefore, many studies have investigated the development of 3D culture systems to better mimic the *in vivo* myocardium conditions, its extracellular microenvironment and cell-cell interactions, to allow biochemical and physical stimuli to distribute across the tissue evenly with the aim to promote maturation (Silbernagel et al., 2020). Typically, current 3D culture models contain a scaffold matrix such as a hydrogel which is then populated with cells to form a strip of contracting micro-tissue creating an engineered heart tissue (EHT) (Roshanbinfar et al., 2019; Tan et al., 2015). Alternatively, 3D cultures of smaller cellular aggregates (spheroids) created without the use of a scaffold protein in hanging drops have also been used for drug testing and toxicology studies (Richards et al., 2016).

Evidence from previous studies shows that 3D cultures of iPSC-CMs encourage structural, functional and metabolic maturation (Ronaldson-Bouchard et al., 2018). hESC-CMs and iPSC-CMs cultured in a 3D matrix showed that uniaxial mechanical stretch increased myocyte alignment, myofibrillogenesis,

sarcomeric banding, hypertrophy and increased force production (Mills et al., 2017). As a result of improved myofibril alignment and developed sarcomere, iPSC-CMs display better calcium handling (Klein & Ojamaa, 2001; Krüger et al., 2008; Li et al., 2014).

Although 3D cultures have great potential to encourage maturation of iPSC-CMs, it may be insufficient on its own, thus, several studies use a combination of 3D methods with other techniques, such as electrical or physiological stimulation. One such example is when iPSC-CMs were embedded with collagen into PDMS channel to form a wire-like structure which was then exposed to electrical stimulation which encouraged functional maturation. Incorporating electrically conductive silicon nanowires or carbon nanotubes into the channels formed an electrically conductive environment and augmented electrical conduction (Rodriguez, et al., 2014). The cells in the microtissue were then exposed to exogenous electrical stimulation to provide a biomimetic pace-maker-like stimuli to enhance the electrical properties of the cardiac constructs (Lee et al., 2010); this improving calcium handling and contraction force (Nakamura & Sadoshima, 2018).

In summary, 3D culture is positively influencing the maturation of iPSC-CMs. The advantage of combining 3D culture with other techniques allows the development of a preferable culture environment to promote the maturation of cells, however, these methods still need further optimisation to reach the desired level of maturity.

1.6.4 iPSC-CMs maturation by biochemical cues

Metabolic and hormonal interventions have emerged over the past 5 years and are crucial regulators of myocyte maturation in terms of gene expression patterns, hypertrophy and cell cycle exit (Troncoso et al., 2014). Therefore, the treatment of iPSC-CMs with additional supplements to modulate hormonal signalling during cell culture may encourage further progress in the field.

Previous literature shows that metabolic hormone levels at birth rise simultaneously with the maturation of cardiac properties. In particular, the rise of the thyroid hormone triiodothyronine (T3) after birth has been noted and is understood to be essential for normal cardiac development and function (Rupert & Coulombe, 2017). The treatment of iPSC-CMs with T3 has shown to increase cell size, sarcomere length and contractile force along with reduced cell cycle activity. Furthermore, T3 treatment enhanced contractile kinetics and calcium propagation, resulting in increased force generation (Yang et al., 2019). A study conducted on murine ESC-CMs showed that treatment with T3 hormone led to the upregulation of the cardiac pace maker genes *Nkx2.5*, *myosin light chain-2v*, α - and β -myosin heavy chain as well as sarco(endo)plasmic reticulum calcium ATPase-2a and ryanodine receptor-2 expression. Furthermore, electrophysiology data showed that T3-treated ESC-CMs displayed a more adult-like calcium handling properties based on increased maximal upstroke and decay velocity as well as larger peak amplitude of calcium transients in response to stimulation with caffeine (Mills et al., 2017).

Additional factors such as insulin-like growth factor-1 (IGF-1) and neuregulin 1 β (NRG1) are also known to promote cardiomyocyte development (Correia et al., 2017) by regulating signalling pathways which control hypertrophy, metabolism and contractility in the heart (Nakano et al., 2017). Treatment of hESC-CMs with NRG1 strongly promotes cell growth and metabolic remodelling with improved force-frequency relationship (Nakano et al., 2017).

The switch from glycolysis to fatty acid oxidation after birth is an important transition of myocyte development and can be mimicked in iPSC-CMs culture by supplementation of fatty acids in the culture medium. The treatment of iPSC-CMs with palmitate, oleate and linoleate as a cocktail of fatty acids has shown to increase cell size, single-cell force production, calcium handling and action potential upstroke velocity (Nakano et al., 2017). A study conducted by Mills et al. screened for an optimal medium for myocyte maturation. Using an iPSC-CMs organoid model they reported that a high palmitate content with low glucose induced a shift towards postnatal gene and protein

expression patterns and increased oxidative phosphorylation and calcium handling properties, thus was the most desirable medium for metabolic, functional and cell cycle maturation (Mills et al., 2017). Fatty acid-rich media has shown to encourage denser cristae development in mitochondria, activate oxidative phosphorylation, increase binucleation, promote sarcomeric maturation which improved contractile force generation (Nakano et al., 2017). Similarly, depriving iPSC-CMs of glucose also positively influences cardiac structural, metabolic and functional properties (Nakano et al., 2017).

To summarise, there are a number of distinct hallmarks of cardiomyocyte maturation, and even though these are well described, there are still some barriers we need to overcome. For example, the molecular mechanisms which mediate change from immature to mature are not fully understood. One method which supports natural development and maturation of iPSC-CMs is through prolonged culture time. Although there are numerous advantages of this method of promoting myocyte maturation, further investigation remains to be conducted.

1.7 HYPOTHESIS AND AIMS

Hypothesis:

I hypothesise that β_2 AR genetic variants play an important role in regulation of receptor-dependent cAMP signalling due to their variable G_s/G_i composition, thus, influencing cardiomyocyte physiology. I hypothesise that iPSC-CMs possess limited and underdeveloped structural microdomains and hence will display an immature and uncoordinated β_2 AR-derived cAMP signalling and PDE activity. I hypothesise that employing a mechanism to encourage controlled, structured growth will enhance the natural developmental process of cardiac microdomains development and alter β_2 AR-dependent cAMP signalling and PDE activity.

Aims:

The principal aim of this project is to investigate how in vitro methods of caveolar maturation in iPSC-CM may develop cAMP signalosomes, thus influencing temporal and spatial activity of β_2 AR-generated cAMP and PDE activity. The three mechanisms of structural and functional maturation to be investigated are: 1) Cav β 2 mimetic peptide administration, 2) microcontact printing, and 3) time-dependent maturation in culture

The aims of this project are broken down into the following objectives:

1. Investigate the role of the β_2 AR haplotypes on receptor activity

- Distinguish the influence of β_2 AR haplotypes on cAMP activity
- Identify the β_2 AR haplotypes influence on cardiomyocyte beating rate
- Investigate the role of β_2 AR- G_i coupling properties during stress induction

2. Investigate how maturation through Cav β 2 mimetic peptide administration may influence T-tubule domains development and LTCC localisation and functionality

- Assess the effect of peptide administration of T-tubule associated gene expression
- Investigate its influence on structural development through assessing surface topography
- Assess LTCC activity

3. Investigate how manipulation through micropatterning matures iPSC-CMs development

- Develop a protocol for micropatterning iPSC-CMs
- Assess the influence of micropatterning on myofibril development
- Assess the influence on functional development through β AR signalling and PDE activity

4. Identify the role of microdomains in regulating β 2AR-cAMP signalling and PDE activity in iPSC-CMs

- Depict β 1AR and β 2AR expression and cAMP signalling ratio
- Assess β 1AR and β 2AR-dependent cAMP signalling within caveolae
- Assess the role of caveolae on PDE2, 3, and 4 activity

5. Identify the effect of ageing iPSC-CMs as a method of maturation by investigating the influence on β 2AR-cAMP signalling and PDE activity in iPSC-CMs

- Quantify the effect of ageing on caveolae compartments
- Assess how β AR-dependent cAMP signalling and PDE2,3,4 activity within caveolae change over time

CHAPTER 2: METHODS AND MATERIALS

2.1 CELL CULTURE

Three cell lines were used in this project. Firstly, an IMR-90 cell line of human induced pluripotent stem cell (hiPSC) (WiCell Technologies). These were differentiated in-house into cardiomyocytes using the protocol described below (Section 2.1.1). Secondly, the Cellular Dynamics International (CDI) hiPSC-CMs cell line was used as an alternative iPSC-CMs line for comparison with IMR-90. The third cell line, human embryonic stem cell-derived cardiomyocytes (HUES7 hESC-CMs) were a kind gift from and made in Chris Denning's lab, Nottingham University, and were transported to Imperial College London. They were genetically manipulated using CRISPR/Cas9 conducted by Alexander Kondrashov in Chris Denning's lab, to produce isogenic cell lines of the β_2 AR haplotypes.

2.1.1 iPSC-CMs (IMR90) generation

The IMR-90 human iPSC cell line (WiCell Technologies) was derived from human Caucasian foetal lung fibroblasts (J. Yu et al., 2007) from which cardiomyocytes were generated in-house by Thusharika Kodagoda using small molecule activators and inhibitors of the Wnt signalling pathway (B. Li et al., 2017; Lian et al., 2013). All products are from Life Technologies (UK) unless otherwise stated. Generally, iPSCs were grown on 6-well, plastic-bottom plates which were coated with Matrigel (1:50) for at least 1 hour at 37°C prior to cell plating. iPSCs were cultured in Essential-8 media (E8) until they were approximately 80-90% confluent, media was refreshed every day. To begin differentiation (at D0), 8 μ M Wnt-activator CHIR99021 in Roswell Park Memorial Institute medium (RPMI) (Gibco) + 1X B27 minus insulin (RB-) was added to the cells for 48 hours. On D2, media was changed back to RB-. On D3, media was aspirated and cells were then treated with RB- with C59. On D5, media was changed back to RB-. On D7, metabolic selection was conducted by the depletion of glucose to remove non-cardiomyocytes which provided an hiPSC-CM purity of >90%. Contracting cells would occur around D7. On D15, cells were then returned to regular RPMI + 1X B27 for 1-2 days and were ready to be dissociated and replated (fig 2.1).

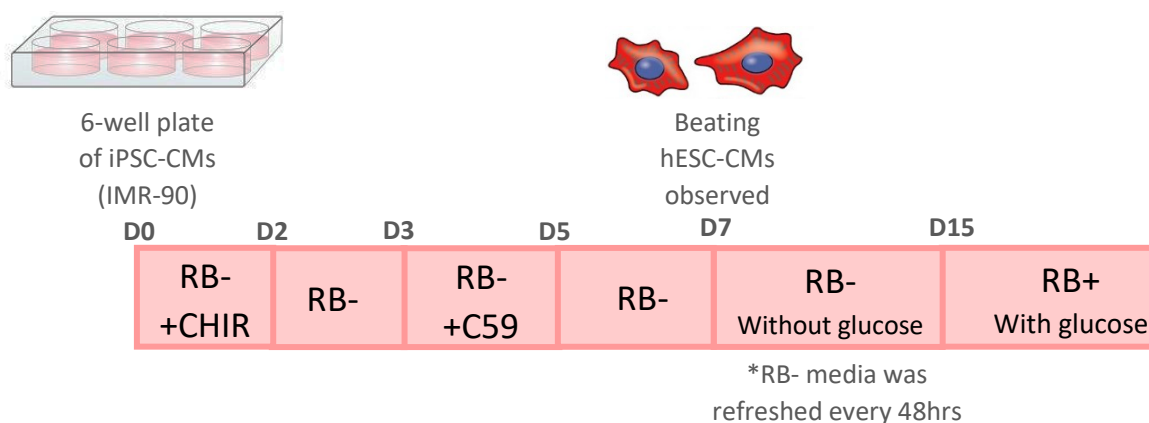


Figure 2. 1 Illustration of (IMR-90) iPSC differentiation protocol into iPSC-CMs

Differentiation was conducted over a period of 15 days with the respective media and used for experimentation at either D30, D60 or D90 as required.

2.1.2 iPSC-CMs (IMR90) dissociation and maintenance

Cells were then dissociated and re-plated in the following way. Culture media was aspirated and iPSC-CMs at D15-D16 were washed by incubating with 2-3mL phosphate buffered saline (PBS) for 4 mins at room temperature (RT) before incubating in cell dissociation solution (CDS) (containing 40 % cell dissociation buffer (Invitrogen), 40 % RPMI basal medium (Gibco), 20 % of 0.05 % Trypsin-EDTA (Gibco). 1mL CDS was added to each iPSC-CM well of the 6 well plate and incubated at 37 °C for 8 minutes. The solution was then pipetted up and down to dislodge the cells from the well and break up any large cell clusters and transferred to a 15mL falcon. 1mL RPMI+B27+10% FBS was added to the falcon to inactivate the enzymes, and again thoroughly pipetted. A further 2mL RPMI was used to detach any remaining cells from the culture well and added to the falcon. The Falcon tube was spun at 300g for 4 min. Supernatant was aspirated and the pellet resuspended in RPMI + B27 + 10% FBS, and 1% Rho-associated protein kinase (ROCK) inhibitor. 1µL of the cell suspension was stained with acridine orange/ propidium iodide stain (Logos Biosystems) and counted on a Logos Luna dual fluorescence cell counter. Cells were re-plated on Vitronectin-coated plastic-bottom 6-well plates at the density of 1.2×10^6 cells per well and culture media changed after 24h to RPMI + B27 medium.

Media was replaced every 2-3 days until they reached D30-D35. At day 30-35 cells were dissociated again and plated as required for experimental use.

2.1.3 Caveolin-3 overexpression of IMR-90 cell line

The RFP-tagged caveolin-3 plasmid (pCav3) was used to overexpress the Cav3 protein in iPSC-CMs of the IMR-90 cell line. Cells at D30 were transfected with pCav3 using Lipofectamine3000 kit. The manufacturer's guidelines were followed with optimised incubation time. In brief, the Cav3 plasmid DNA (500ng) and 1µl of Lipofectamine3000 Reagent were diluted in 25µl of OptiMEM in separate 1.5mL Eppendorf tubes and incubated at room temperature for 5mins; then were gently vortexed. The plasmid mixture and Lipofectamine3000 mixture were then combined and vortexed gently and incubated for 20 min at room temperature. This was then added into the cell culture dishes containing 1mL of culture media for 48h. Cells were then ready for experimentation.

2.1.4 iPSC-CMs (CDI) plating and maintenance

Human iPSC-CMs were obtained from Cellular Dynamics International (CDI). The 1.5mL cryopreserved single-cell suspension vials were thawed, seeded and cultured following the User Guide protocols as described below.

Culture dishes as required were coated with 0.1% gelatin for a minimum of 15min at 37°C. The gelatin was removed immediately before the addition of cells. The 1.5mL cryovial of CDI's was removed from liquid nitrogen storage tank and thawed at 37°C in a water bath for 4min, ensuring the vial stayed as stationary as possible. Once cells were thawed, they were gently transferred to a sterile 50mL falcon tube in a drop-wise fashion to minimise shock to the cells. The vial was rinsed with 1mL of room temperature iCell plating media to harvest any remaining cells and then also added to the falcon drop-

wise over a 90-second period. The falcon was swirled gently at regular intervals. A further volume of iCell plating medium was added according to the desired seeding density using the equation below (generally, 2.5mL media for a 3×10^4 cells/well plating density). The falcon was mixed thoroughly before plating the cells.

$$\text{Viable cell density} = \frac{\text{viable cells per vial} \times \text{plating efficiency} \times \text{viability}}{\text{Cell suspension volume}}$$

CDI's were plated onto the centre of the pre-prepared, gelatin-coated culture dishes and incubated at 37°C, 5% CO₂. After 48h, the debris and unadhered cells were washed and replaced by 2mL of iMaintenance Medium to each dish (which was every 48h thereforth). As recommended by the manufacturers, cells were cultured for 10 days before use in order to reach maximal stability (fig2.2).

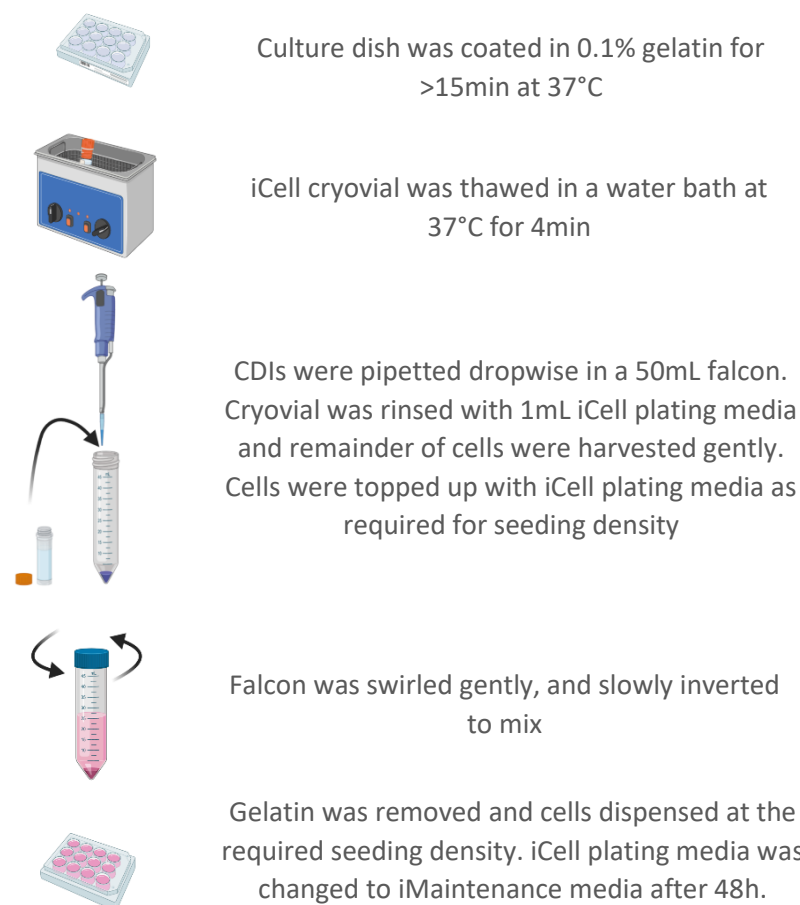


Figure 2. 2 CDI (iPSC-CMs) plating and maintenance protocol

2.1.5 hESC-CMs generation and maintenance

2.1.5.1 Development of genetically engineered hESC-CMs lines by CRISPR/Cas9

The development of the isogenic hESC-CMs lines for studying β_2 AR haplotypes were produced by Alexander Kondrashov in Chris Denning's Lab at Nottingham University and were a kind gift for this project. The 4 isogenic homozygous variants were created from the human embryonic stem cell line (HUES7). A new method developed by Alexander Kondrashov combines the CRISPR/Cas9 and *PiggyBac* approaches in a two-step strategy which shortened the editing duration by 2 weeks and only required one round of clonal selection, thus making this method more efficient (Kondrashov et al., 2018). This method is also footprint-free and so genome modifications post-editing can be prevented.

This method uses targeting vectors which contained the β_2 AR polymorphic variants of interest and a positive-negative drug selection cassette flanked by the *PiggyBac* transposon recombination sites. These components are flanked by regions homologous to the endogenous target locus of around 1kb in length; this allows recombination between the template and the genome. After the transfection of HUES7 hESCs with the ADRB2 targeting plasmid (the selection vector), Cas9 and gRNA, the cell culture was treated with puromycin. Drug resistant clones were selected and a small sample genotyped. The remainder were harvested and then transfected with transposase to excise the selection cassette by the recombination of *PiggyBac* sequences which restructured the locus and cells were now insensitive to ganciclovir. This allowed the successful production of an isogenic set of cell lines containing the four β_2 AR variants (GE, GQ, RQ, RE) which were verified by PCR genotyping and sequencing.

2.1.5.2 hESC differentiation into cardiomyocytes

Once the four β_2 AR variants of hESCs were developed, as described in section 2.1.6.1, they were differentiated into cardiomyocytes in the following way. The differentiation was conducted by Alexander Kondrashov at Nottingham University. The transgenic lines of hESCs were initially seeded at 40,000 cells/cm² in a Matrigel-coated T25 flask. Cells were cultured in 10mL E8 media at 37°C for approximately 3-5 days until confluency was reached, media was refreshed every day to prevent accumulation of debris and prevent hESC dedifferentiation. The culture was then pre-conditioned with StemPro34 media containing 1 ng/mL BMP4 and Matrigel (1:100) 24h before initiating differentiation. Once 80-90% confluency was reached, D0, cells were treated with StemPro34 media containing 10ng/mL of BMP4 and 8ng/mL of Activin-A for 48h. On D2, media was aspirated and switched to RPMI + B27(minus insulin) with 10 μ M of KY02111 and XAV939 (KYX media). On D4, media was refreshed to the same RPMI + B27 (minus insulin) and 10 μ M of KYX media. On D6, beating cells could be seen, and the media was replaced with RB27 in which the cells continued to be maintained from this point onwards, media was refreshed every 48h until D15. At D15, hESC-CMs were dissociated and re-plated onto fibronectin-coated, plastic T25 flasks and cultured in RB+ medium (Fig 2.3).

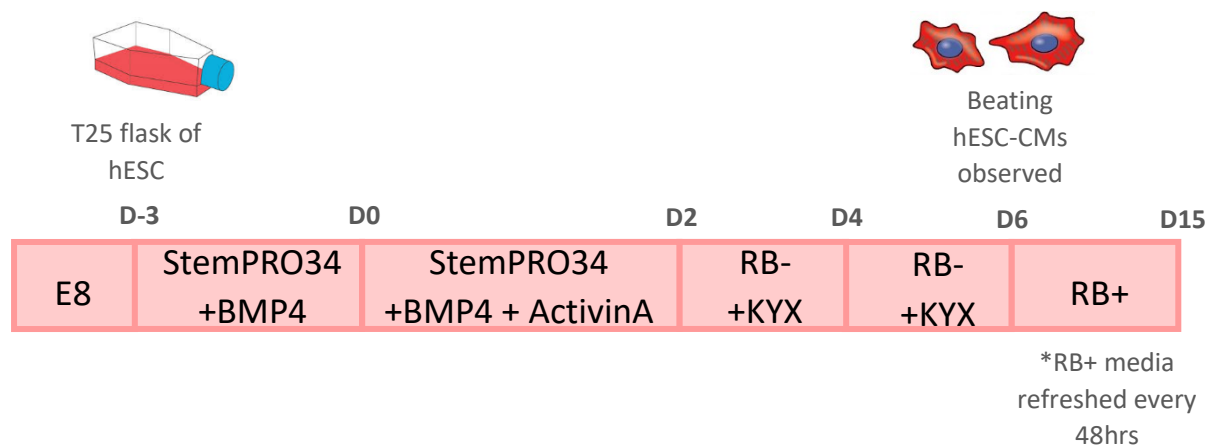


Figure 2. 3 Illustration of hESC differentiation protocol into hESC-CMs

Differentiation was conducted over a period of 15 days with the respective media and used for experimentation around D30.

2.1.5.3 Cell culture and dissociation

hESC-CM were plated on plastic-bottomed T25 culture flasks where obtained from Nottingham around D15-D20 and maintained in RPMI + B27 medium at Imperial College London until D30. When aged to Day 30-35, they were ready to be dissociated and replated as required for experimentation. Firstly, the culture medium was aspirated, and cells were incubated in 5ml PBS for 4min at room temperature (RT) before incubating in 3ml cell dissociation solution (CDS) (consisting of 40% cell dissociation buffer, 40% RPMI basal media and 20% Tryple E select) for 8min at 37°C. 3ml RPMI+B27+10% FBS was added to the flask to inactivate the enzymes, and thoroughly washed to detach cells. Cells were collected, dispensed into a Falcon tube. A further 3mL RPMI+B27+10% FBS was used to wash the flask again to collect any remaining cells. The falcon was spun at 300g for 4min. Supernatant was aspirated and pellet resuspended in RPMI + B27 + 10% FBS. Cells were counted and plated at the required density on Vitronectin-coated Glass-bottom MatTek dishes for experiments. Cells were cultured in BR+ until D30-D35.

2.2 MANIPULATION OF SC-CMS MATURATION

Three methods of maturation were explored in this project. The IMR90 iPSC-CMs cell lines were used for all methods. For each method, the same differentiation and culture protocols were used as described in sections 2.1.1 and 2.1.2.

2.2.1 Long term culture

For studies conducted in aged cells, iPSCs were differentiated into cardiomyocytes as usual (outlined above) and iPSC-CMs at D30 were re-plated into 6-well plastic bottom culture dishes. Cells were maintained in RPMI+B27 media at 37°C with 5% CO₂, and media was changed every 3 days until they reached 90±10 days. These were then dissociated and replated as described in Section 2.1.2 into MatTek dishes or coverslips as required.

2.2.2 Cell patterning and alignment

Poly-di-methyl-siloxane (PDMS) stamps were produced by Ravi Desai at Francis Crick Institute and were a kind gift. They were designed to have grooves of 20µm in width and 20µm in depth as shown in Fig 2.4. Before each use, the PDMS stamps were cleaned by sonication in 100% ethanol for 10min, then rinsed with 70% ethanol and finally rinsed in distilled water. The stamps were then dried with N₂ from a compressed N₂ source fitted with a handgun. The stamps were placed patterned side up and loaded with 200µL of 0.1% fibronectin (Sigma). These were carefully placed in 10x10cm dishes and transferred into an incubator with particular care to ensure the bubble of matrix remains on the stamp. These were incubated at 37°C overnight. The stamps were removed from the incubator and gently rinsed with sterile H₂O ensuring no direct pressure of water was applied to the face of the stamp. Once gently rinsed, the stamps were dried with N₂. Immediately after, stamps were inverted

and pressed firmly onto a glass-bottom culture dish (Ibidi) and left in contact for 30min at 37°C. After incubation, the stamps were gently peeled away from the culture dish and 500μL of 0.5% Pluronic F127 (w/v) (Sigma Aldrich) in H₂O was added and again incubated for 30min 37°C. Pluronic was aspirated and the dish was gently washed with PBS once before seeding the cells (fig 2.4) (Desi et al., 2014).

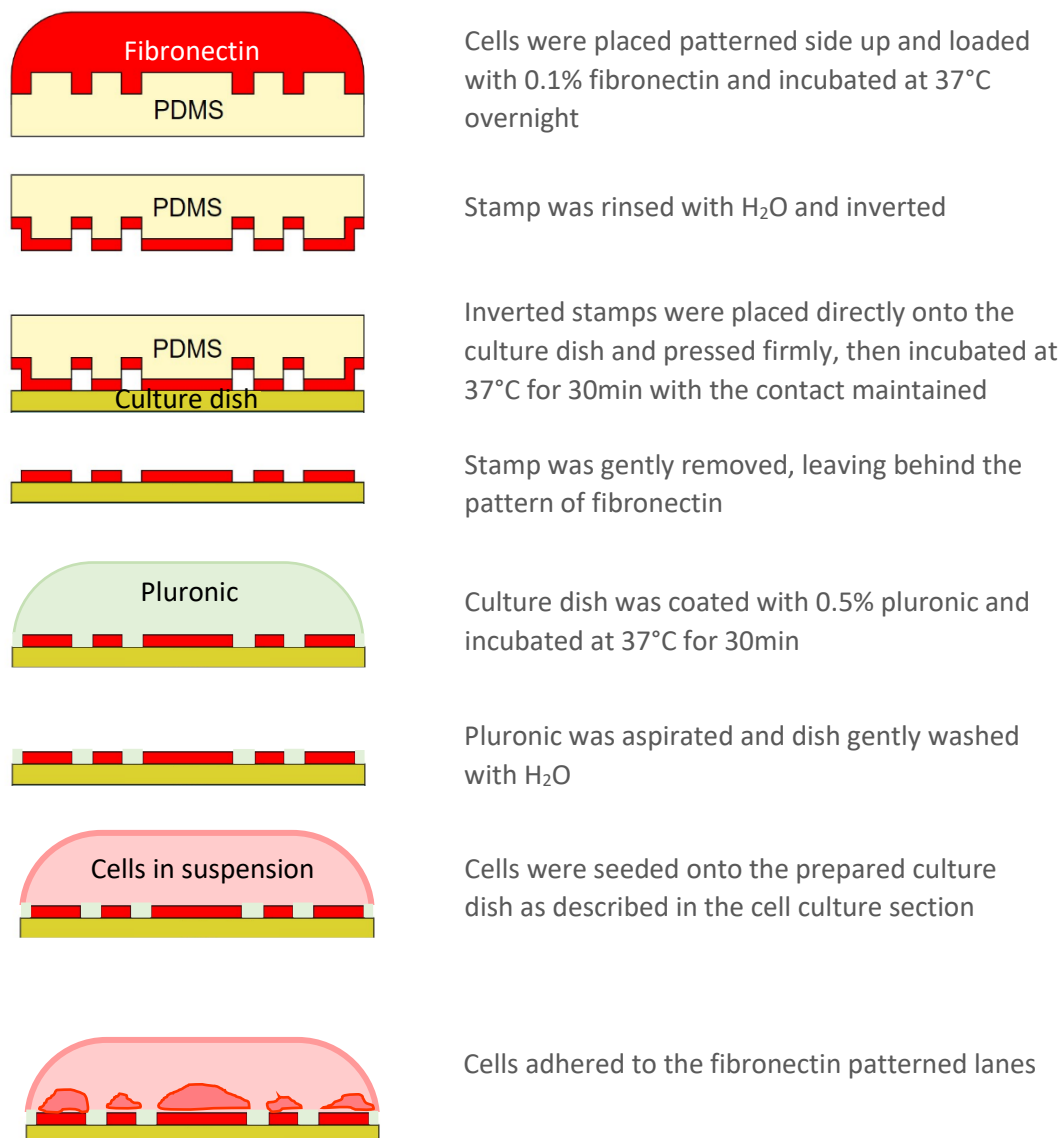


Figure 2. 4 Protocol for preparing dishes for microcontact printing

Schematic depicting the process of producing culture dishes for patterning cells using PDMS stamps.

The PDMS stamps are 1 x 1 cm².

2.2.3 Transfection with $\text{Ca}_v\beta 2$ memetic peptide

To transfect cells with Scramble (SCR) and Mimetic peptide (MP), 1.3 μM of the peptide was added to the RPMI culture medium. The IMR cells were then incubated in this media for 4 days at 37°C. If medium was changed, fresh peptide was added to the medium (fig 2.5). The peptides were a kind gift from Professor Daniele Catalucci from Italy (Rusconi et al., 2016). The MP peptide was added at specific time points throughout differentiation; at D5, D10, D20 and D30. The experiment was designed to have a SCR control group for each MP treatment group. Unfortunately, due to the departure of a collaborator, an SCR control was only obtained for the D5 group and the D10, D20 and D30 could not be completed.

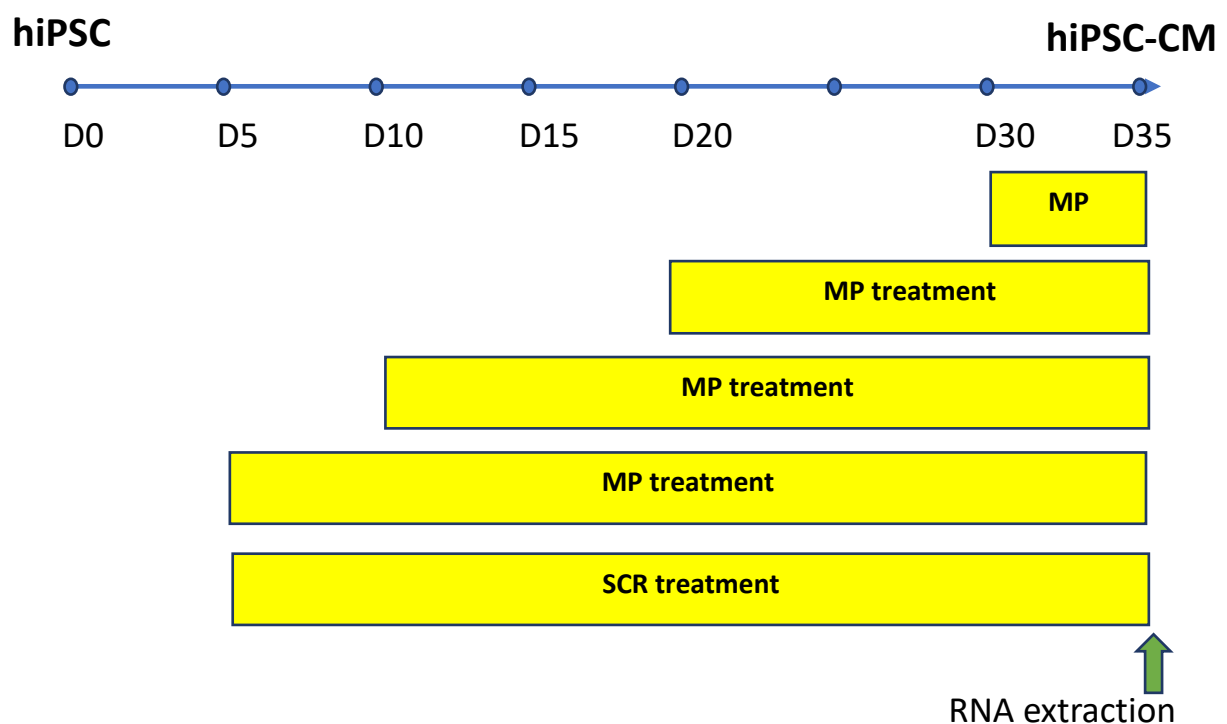


Figure 2. 5 MP and SCR treatment ptotocol

Protocol depicting the time-points and the duration of MP and SCR treatment of iPSCs throughout the differentiation period into iPSC-CMs. All cells were cultured until D35 at which point they were harvest and prepared for RNA extraction.

2.3 RT-QPCR

2.3.1 RNA Extraction

RNA was extracted using the RNeasy Mini kit (Qiagen, CA, USA) from cultured hiPSC-CMs according to the manufacturer's guidelines in the following way. The cell culture media was removed from the culture dishes and the cells were washed with PBS. 700 μ L of TRI-Reagent (Sigma-Aldrich) was added and cell scraper was used to dislodge the cells. Cells were harvested and collected in a 2mL Eppendorf to which 700 μ L of 70% ethanol was added. This was pipetted up and down to mix well and then transferred to an RNeasy mini-spin column. The column was spun at 800g for 30 seconds, the flow-through was discarded. A preparation of cold (maintained in ice) 10 μ L DNase1 stock (Qiagen) in 70 μ L RDD buffer was added to the mini-spin column membrane and incubated for 15min at room temperature. 350 μ L RW1 buffer was added to the column and spun at 800g for 30 seconds and flow-through discarded. 500 μ L of RPE buffer was added to the column and spun under the same conditions and the flow-through discarded. The column was transferred to a new 2mL flow-through tube and spun for a second time at max speed for 2min to dry the membrane of the column. 32 μ L of nuclease-free water was added directly to the mini-spin column and incubated at room temperature for 10min. The column was placed in a sterile Eppendorf and spun at 8000g for 2min. Total mRNA concentration was measured using NanoDrop UV-Vis Spectrophotometer.

2.3.2 RT-QPCR protocol

Gene expression profiles were investigated in iPSC-CMs as described in each specific chapter. Firstly, cells were harvested and RNA was extracted as described above. The Qiagen kit was used to perform RT-QPCR. The RNA samples, primers and SYBR-Green were thawed in ice and slightly vortexed to ensure the reagents and samples were mixed well. A mastermix containing 187.5 μ L SYBR green fluorescence marker (Qiagen), 3.75 μ L forward primer, 3.75 μ L reverse primer (Sigma-Aldrich), 3.75 μ L of

reverse transcriptase (Qiagen) and 30µl of nuclease-free water was added and gently but thoroughly pipetted. 15.25µl of the mastermix was dispensed into each well of a 96-well plate. To each well 9.75µl of 1ng/µl of each RNA sample or nuclease-free water (for negative controls) was added. The plate was sealed, then gently vortexed for thorough mixing and centrifuged to remove any air bubbles. RT-QPCR took place using the Eppendorf RealPlex MasterCycler (Thermo Fisher). All QPCR reactions were carried out in triplicates. The data was normalised to the housekeeping gene GAPDH. The fold differences were calculated through using the “ $2^{-\Delta\Delta Ct}$ ” method.

Target Gene	Forward sequence	Reverse sequence
β_1 AR	GACGCTCACCAACCTCTTCA	CACAGCTCGCAGAAGAAGGA
β_2 AR	TTGCCTCTTCCATCGTGTC	CCACCTGGCTAAGGTTCTGG
CAV3	GCGGAAGGAGGTCTAAAGC	AGAGCGAAGGGCCATTGT
PDE2A	CCTCCTGTGACCTCTCTGACC	TGAACTTGTGGGACACCTTGG
PDE3A	TCACAGGGCCTTAACCTACAC	GGAGCAAGAATTGGTTTGTCC
PDE3B	CCTCAGGCAGTTTTATACAATG	TGCTTCTTCATCTCCCTGCTC
PDE4A	GTGGAGAAGTCTCAGGTGGG	TGGAAGTGTGAGGCAGGG
PDE4B	TAGAAGATAACAGGAAGTGG	GCAATGTCTATGTCAGTCTC
PDE4C	TGATCTGAGCAACCCACCAAG	GGTGAGGTCTGAGGGACTTCG
PDE4D	GGATAATGGAGGAGTTCTTCC	CGATTGTCCTCCAAAGTGTC
BIN1	GCAGGCTCATCTCGTAGCTC	AAACACCTTCTGGGCTTTGA
cTnT	GGCAGCGGAAGAGGATGCTGAA	GAGGCACCAAGTTGGGCATGAACGA
α -ACTININ	TCCCTGGAAAAGAGCTACGA	TGCTGTTGTAGGTGGTCTCTCG
JPH2	GACATCGCGAGAGCTGTG	TTCCTGAAATCTCTGTTTGACG
GAPDH	AATCCCATCACCATCTTCCA	TGGAAGTCCACGACGTACTCA

Table 2. 1 Table of forward and reverse primer sequences for the respective genes

The program for PCR cycles program was as follows (fig 2.6): 95°C (2min) and then 40 cycles with 95°C (15s), 55°C (1min) and 68°C (20s).

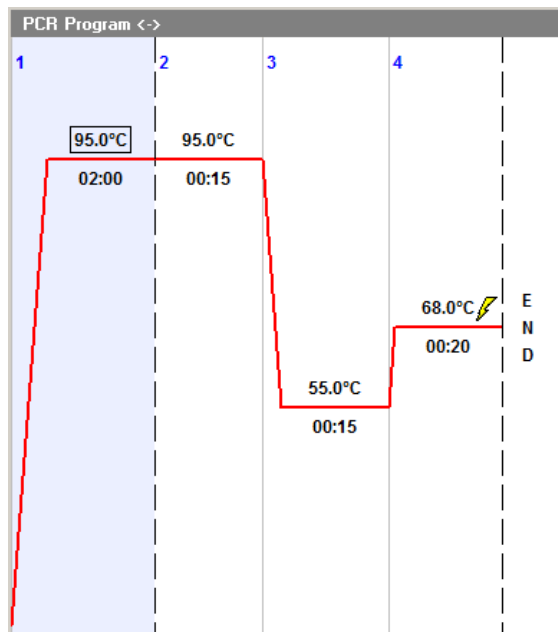


Figure 2. 6 RT-QPCR program

The RT-QPCR program used, where stages 2-4 were repeated for 40 cycles.

2.4 TEM

2.4.1 Reagents and protocols

Culture medium was removed from cell culture dishes; cells were rinsed with PBS to remove any unadhered cells and debris then fixed in 2.5% glutaraldehyde in cacodylate buffer. Further preparation was conducted by Ivan Diakonov in Professor Julia Gorelik's group. In brief, the cells were scraped off the culture dish and placed in a 2mL tube and centrifuged to form a pellet. Warm 2% agarose (w/v) was added to the tube and allowed to form a gel around the cell pellet, then an additional 500µL of glutaraldehyde was added and the sample was left overnight. The sample then was post-fixed with osmium tetroxide, processed according to a standard protocol and embedded in Araldite. Sections were cut from the hardened sample and stained with Methylene blue then trimmed around the areas containing the cells. Then 0.1µm sections were obtained and stained with uranyl acetate and lead citrate to be visualised with a transmission electron microscope. In the case of determining the effect of depleting caveolae, cells were treated with 1-2mM MβCD and incubated at 37°C for 1h before fixation.

Caveolae were categorised as flask-shaped, inward curves of the plasma membrane of about 50nm. Darker membrane invaginations were not counted as these are known to be clathrin-coated pits. Analysis was conducted independently by 3 members of the lab on each set of images. Lab members were blinded to the treatment groups in order to minimise bias.

2.5 IMMUNOSTAINING

2.5.1 Reagents and protocols

Blocking Buffer (20 mL)		Final Concentration	Supplier
BSA	1 g	5%	Fisher
Tween-20	50 µL	0.05%	Sigma
Horse Serum	4 mL	20%	Invitrogen
1x PBS	16 L		

Table 2. 2 Blocking Buffer recipe

10x PBS (1 L) – pH 7.4		Final Concentration		Supplier
Sodium Chloride	NaCl	136.9 mM	80 g	Sigma
Potassium Chloride	KCl	26.8 mM	2 g	Sigma
Potassium Phosphate	KH ₂ PO ₄ – pH 7.2	17.63 mM	2.4 g	Sigma
Sodium Phosphate	Na ₂ HPO ₄ – pH 7.2	10 mM	14.4 g	Sigma
ddH ₂ O	1 L			

Table 2. 3 PBS recipe

Name	Species	Final concentrations	Supplier
a-Actinin	Mouse	1:500	Sigma
cTnT	Rabbit	1:500	Abcam
Caveolin3	Mouse	1:100	Sigma

Table 2. 4 Primary antibodies list

Cells were prepared for immunostaining by seeding them on glass coverslips or MatTek dishes. They were cultured for 3-4 days to allow recovery and then prepared for staining. Firstly, the media was removed and the cells were washed with PBS 3 times for 5min each. They were then fixed with 3.7% formaldehyde solution (F1635, Sigma Aldrich, Poole UK) for 15min at room temperature. The samples were washed again with PBS 3 times for 5min. Cells were permeabilised with 0.5% Triton-X for 15min and then washed and placed cell-side down on 100 μ L-droplets of blocking buffer (PBS+10% FBS) on parafilm for 30min at room temperature. Following this incubation period, primary antibodies were prepared according to Table 2.4. Coverslips were placed cell-side down on 100 μ L droplets of the primary antibody preparation in a similar way to the blocking buffer for 2h at room temperature. In the meantime, secondary antibodies (Alexa-Fluor antibodies obtained from Invitrogen) were prepared at a 1:400 dilution together with DAPI (1:1000) in blocking buffer. Coverslips were then washed with PBS 3 times for 10min each and then placed cell-side down in 100 μ L droplets of the secondary antibody preparation at room temperature for 1-2h. Coverslips were then rinsed with PBS and mounted on glass slides (Thermo-Fisher) using mounting solution (Vectashield) and were ready for confocal microscopy.

2.6 FRET MICROSCOPY

2.6.1 cAMP measurements using FRET microscopy

Förster resonance energy transfer (FRET) allows the real-time visualisation of molecular interactions. The phenomenon FRET is based on the postulation that when two light-sensitive chemical compounds (fluorophores) have partially overlapping excitation and transmission energies, they undergo a certain degree of resonance based on their proximity. This is through the transfer of energy from the donor fluorophore upon excitation to the acceptor fluorophore. Due to the sensitivity to proximity, conformational shifts due to cAMP binding to PKA means that the change in fluorescence ratios would semi-quantitatively indicate cAMP concentrations. This initiates the development of genetically-encoded fusion peptides comprising of two fluorophores and a sensor domain. In such a way, upon the binding of a cyclic nucleotide molecule to its respective binding site on the FRET sensor to study cAMP activity has become a popular approach (Börner et al., 2011; Niino et al., 2009).

2.6.2 FRET biosensors

Many types of genetically encoded, FRET-based biosensors have been developed to image the spatiotemporal dynamics of intracellular molecules. The general design of a FRET-based biosensor is illustrated in Figure 2.7. Upon the binding of a small molecule to the “sensing domain”, the structural change of the FRET sensor and hence the increased distance between the fluorophores results in a decreased ratio of acceptor to donor intensity. This correlates with a lower FRET efficiency (Carlson & Campbell, 2009). The FRET sensor, mTurquoise-Epac1-Venus-Venus (pTEPACVV; pTEV) (Klarenbeek et al., 2011) was transfected into iPSC-CMs and hESC-CMs as described in section 2.6.5-2.6.7. Compared to the traditional EPAC2-based sensors, pTEV has an improved cAMP-binding domain with more sensitivity and selectivity; as well as improved fluorescent proteins as donor and acceptor fluorophores (Fig 2.7).

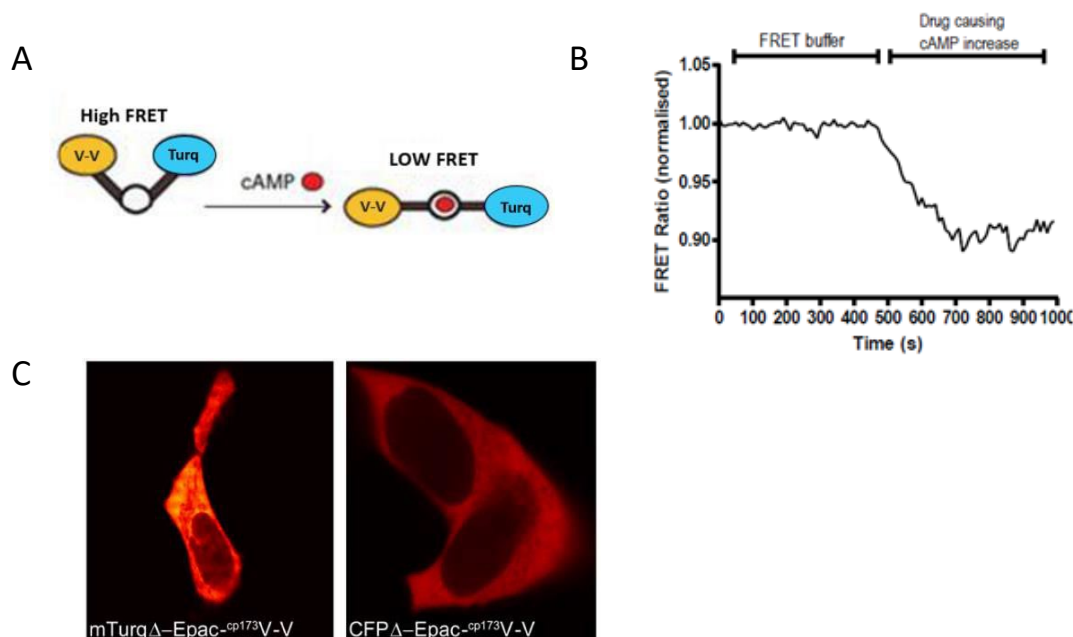


Figure 2. 7 Illustration of pTEV FRET sensor and transfection intensities

A) Illustration of the conformation change of the sensor induced by cAMP binding (adapted from (Börner et al., 2011)). B) An example of a FRET response after the addition of a cAMP-increasing drug. The FRET ratio corresponds to acceptor/donor. C) Illustration of cells expressing the respective constructs throughout the cytosol 18h after transfection. With the same image acquisition properties, it demonstrates greater intensity of fluorescence after the replacement of CFP with Turquoise protein (Klarenbeek et al., 2011).

2.6.3 System setup – Quadview

FRET microscopy was performed as described in Wright et al. (2014). The FRET system consisted of an ORCA-ER CCD (Hamamatsu Photonics) camera attached to an inverted Nikon TE2000 micro-scope, which had a 100W halogen lamp illuminator and EX436/20 excitation filter. The fluorescent light emitted from the object was split into YFP and CFP channels by Quad view beam splitter equipped with a DM455 dichroic mirror and 535/40 and 480/30 emission filters. The recorded images were analysed by quantifying the relative ratio of YFP to CFP using Micro-Manager1.4 software package.

2.6.4 System setup – High throughput, MultiFRET

The FRET system was upgraded during this project and a new software for conducting FRET microscopy was designed and set up by Masoud Ramuz, a PhD student in Professor Julia Gorelik's group. A few upgraded modifications were put in place. Firstly, MultiFRET used a motorised microscope stage making localisation of cells much easier and faster when the acquisition was run. Secondly, the location of individual cells positions (X, Y, Z co-ordinates) were recorded prior to the start of an experiment which allowed each image to be in focus with the cell centred for each image captured. Furthermore, multiple single-cell recordings to be conducted in one experimental setup within the same dish allowing higher throughput. It used an updated Java Plugin within Micro-Manager (Icy) which made real-time calculations of the ratio-metric signals as the acquisitions were taken (fig 2.8) (Ramuz et al., 2019), unlike the previous system where ratio calculations were performed manually.

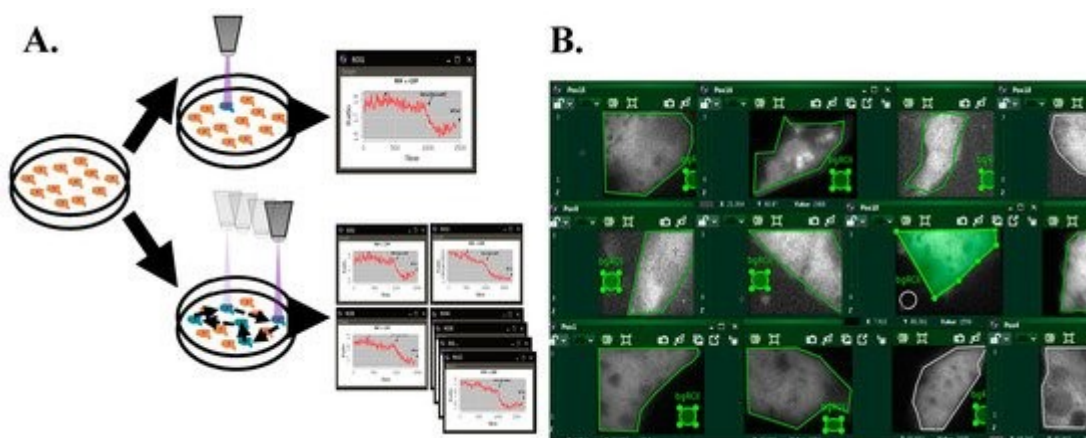


Figure 2. 8 MultiFRET system upgrade

A) High throughput multiFRET microscopy improves on the old method of single cell analysis, top, to the new software, bottom. B) Multiple cells or regions of interest within the same dish/experiment can be selected for analysis at one time (Ramuz et al., 2019).

2.6.5 Transfection of iPSC-CMs (IMR-90) with pTEV FRET plasmid

After differentiation and culture of iPSC-CMs, cells were dissociated and plated into 7mm glass-bottom MatTek dishes. If experiments were prepared for D30 cells, iPSC-CMs were dissociated around D28 and cultured for 2 days in MatTek dishes in RPMI+B27 media for recovery prior to transfection with the FRET sensor. In a similar way, if experiments were prepared for D90 cells, iPSC-CMs were dissociated around D88 and cultured for 2 days in MatTek dishes prior to transfection. Lipofectamine3000 (ThermoFisher) was used to transfect iPSC-CMs (IMR-90 cell line) with the TEV plasmid (pTEV). A mastermix was made based on the number of dishes being transfected where each dish would receive 50µL Opti-MEM (Thermo Fisher), 1.5µL Lipofectamine, 1.5µL Reagent P3000 and 1µg plasmid DNA. To achieve this, two separate, sterile Eppendorfs were prepared. The first Eppendorf contained 25µL Opti-MEM and P3000 Reagent, and the second Eppendorf contained 25µL Opti-MEM with the plasmid DNA and Lipofectamine. The Eppendorfs were incubated for 5min at room temperature before gently, yet thoroughly, combining and incubating at room temperature for 15min. The total of 50µL of the prepared Lipofectamine mastermix was added directly to the MatTek culture dishes containing fresh culture media and were incubated at 37°C for 2-4 days before use for FRET experiments (fig 2.9).

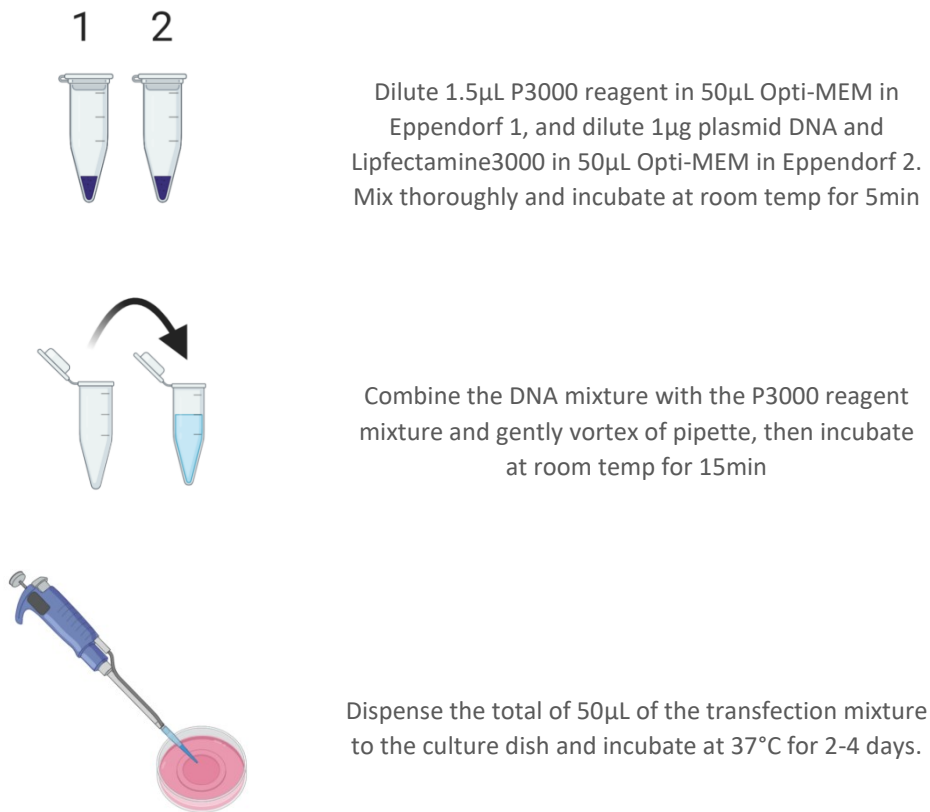


Figure 2. 9 Illustration of the protocol used for the transfection of iPSC-CMs (IMRs)

2.6.6 Transfection of iPSC-CMs (CDI) transfection with pTEV FRET plasmid

The jetPRIME (Polyplus) transfection protocol was used to transfect CDI iPSC-CMs with the cAMP-specific FRET sensor pTEV. To prepare the transfection mixture, in a 1.5mL Eppendorf tube, 2μg of pTEV plasmid was diluted in 50μL of room temperature jetPRIME buffer per MatTek dish to be transfected. This was then vortexed gently for 10sec. To this, 2μL of jetPRIME reagent was added then vortexed gently for 15s before incubating at room temperature for 10min. The 50μL transfection mixture was added to the centre of the MatTek culture dishes on top of the cells in and incubated for 4h at 37°C. The media in the dish was topped up with 1.5mL of CDI iMaintenance medium and incubated for a further 24-72h before use (Fig 2.10).

2.6.7 Transfection of hESC-CMs (HUES7) with pTEV FRET plasmid

For FRET experiments, hESC-CMs were plated on glass-bottomed MatTek dishes at D30-32 and maintained in RB+ for a further 3-5 days to allow cell recovery. At around D35 cells were transfected with the pTEV FRET sensor using JetPRIME (Polyplus) transfection kit following manufacturer's protocol. The pTEV plasmid was thawed at room temperature, and the jetPRIME buffer and jetPRIME reagent were warmed at room temperature. Per dish to be transfected, 200ng of the pTEV plasmid was diluted in 50 μ L of jetPRIME buffer and vortexed on a low speed for 10sec. To this 2 μ L of jetPRIME reagent was added and gently vortexed for 15sec before incubating at RT for 10min. The 50 μ L of transfection mixture was added to the cells in culture dishes containing 1.5ml culture media (RPMI + B27) and incubated for 24-72h before using for FRET experiments (Fig 2.10).

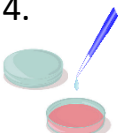
1.  Combine pTEV DNA + 50 μ L JetPrime Buffer
2.  Vortex on low speed for 10-15 seconds
3.  + 2 μ L JetPrime Reagent and vortex for 15 seconds
5.  Incubate for 10 minutes at room temperature
4.  Add transfection mixture to cells and incubate for 24-72 hours

Figure 2. 10 transfection protocol of iPSC-CMs (CDIs) and hESC-CMs (HUES7) with FRET sensor

2.6.8 Reagents and protocols

For all FRET experiments, culture dishes with a maximum volume of 2.5mL were utilised so the addition of succeeding solutions to the FRET buffer was possible. FRET solutions were applied directly to the cells in the bath of the culture dish using a hand-held pipette. All stated drug solutions are mentioned as the final drug concentration in the cell bath. All reagents were made in FRET buffer (Table 2.5).

FRET buffer 2 L - pH (7.4)		Final Concentration		Supplier
Sodium Chloride	NaCl	16.83 g	144 mM	Sigma
Potassium Chloride	KCl	0.746 g	5 mM	Sigma
Magnesium Chloride	MgCL ₂	2 mL	1 mM	Sigma
2[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulphonic acid	HEPES	4.76 g	10 mM	VWR
dH ₂ O		2 L	up to 2 L	

Table 2. 5 FRET buffer recipe

FRET buffer was freshly prepared every 3-4 weeks and was maintained at 4C. It was warmed to room temperature when required for use.

In brief, the following experimental protocols were established to study β AR-dependent cAMP responses to stimulation with isoprenaline (ISO). Detailed methods are described in individual chapters.

2.6.8.1 Isoprenaline dose response curves

Dose responses for β AR stimulation with isoprenaline was conducted to assess the EC₅₀ of both β_1 AR and β_2 AR subtypes. Concentrations ranging from 0.1nM to 100 μ M of ISO were prepared in FRET buffer. CGP20712A (CGP) (Sigma Aldrich, Poole UK) (100nM) was used for blocking β_1 AR and ICI118551 (Tocris, UK) (50nM) was used for blocking β_2 AR prior to ISO stimulation. Upon reaching a plateau in

the FRET response, cells were perfused with NKH477 (NKH) (Sigma Aldrich, Poole UK) (10 μ M) to fully stimulate all AC activity and achieve maximal cAMP release.

	Log KD β_1 AR	pA2 β_1 AR	IC ₅₀ for β_1 AR	Log KD β_2 AR	pA2 β_2 AR	IC ₅₀ for β_2 AR	Selectivity β_2 AR vs. β_1 AR
CGP20712A	-8.81	9.43- 8.58	0.7nM	-6.11	-	6700nM	0.002
ICI 118551	-6.52	-	37.5nM	-9.26	7.11- 6.16	1.2nM	549.5

Table 2. 6 Pharmacological properties of CGP and ICI

(Baker 2005; Baker 2010; Zwaveling et al., 1996; Horinouchi et al., 2003; Doolet et al., 1986)

2.6.8.2 PDE activity assessment

The activity of β AR-dependent cAMP-degrading PDEs in hiPSC-CMs were of interest throughout this project. After β_1 AR or β_2 AR stimulation with ISO, PDE2, PDE3 or PDE4 were selective inhibited with 10 μ M EHNA, 10 μ M Cilostamide and 10 μ M Rolipram respectively. Once a plateau was obtained, 100 μ M IBMX was added to the bath for non-selective inhibition of PDEs, followed by 10 μ M NKH to fully stimulate all AC activity and achieve maximal cAMP release.

2.6.8.3 Assessment of response after caveolae depletion by M β CD treatment

For the removal of caveolae, cells were treated with M β CD (methyl- β -cyclodextrin) prior to experimentation. 1-2mM M β CD was dissolved in room temperature culture media by vortexing thoroughly. The previous culture media was removed from the MatTek dishes and 1.5mL of the M β CD media was added to each dish for 1 hour. Dishes were treated one at a time with careful timing to ensure cells were not in the M β CD media for more than 1.5 hours as it is known to have a negative influence on cell viability and cause non-specific disruption of β_2 AR and LTCC interaction.

2.7 IONOPTIX- CONTRACTION RECORDINGS

2.7.1 Cellular contraction measurements

The IonOptix system works by imaging the movements of high contrast regions of the cell membranes. Many previous studies have made use of this technology to study modulations of cardiomyocyte cell dimensions during their contractility in response to cellular stimulation. This method tracks the movement of high contrast regions at the edges of cardiomyocytes as they contract. As the cells contract, there is a shortening of the cell compared to its length at baseline. This is referred to as fractional shortening and is used as an indicator of the contractile state of the cell and its contractile function. However, unlike adult myocytes, SC-CMs do not possess the typical rod shape and so the parallel ends of the cell cannot be measured. Therefore, in this work, one of the two edges of the tracker were deactivated. In this way, the movement of a single edge of the hESC-CMs membrane allowed each contraction to be recorded and was depicted by a single peak. The fractional shortening could not be measured, only relative changes in beating rate after a stimulus. In this work, the IonOptix technique was employed to report the physiological effect of the β_2 AR signalling at the cellular level through a mechanical response.

2.7.2 System Setup

Cell contractility was measured using an IonOptix system which consisted of a Nikon TE-200 inverted microscope and a MyoCam CCD Digital Camera (IonOptix Corp) attached to a PC. MatTek dishes were placed on the microscope stage. In the MatTek dish, Krebs solution was warmed and perfused using a peristaltic pump. Through this, the behaviour of β_2 AR on cellular contractility upon stimulation with a number of pharmacological agents was assessed as described below. Contractility recordings were acquired and analysed using the IonWizard software package (2.11).

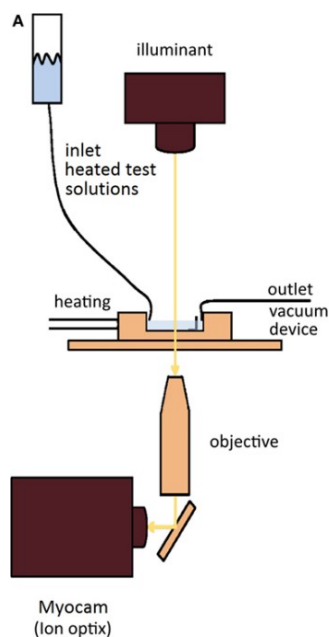


Figure 2. 11 The IonOptix system setup for recording iPSC-CMs contraction

2.7.3 Reagents and protocols

KREBS (1 L) – pH 7.4		Final Concentration		Supplier
D-Glucose		11.5 mM	2.08 g	Sigma
Magnesium Sulfate [Anhydrous]	MgSO ₄	0.94 mM	0.232 g	BDH-GPR
Potassium Phosphate Monobasic	KH ₂ PO ₄	1.2 mM	0.166 g	Sigma
Potassium Chloride	KCl	4.69 mM	0.35 g	Sigma
Sodium Chloride	NaCl	119.1 mM	6.96 g	BDH-AnalaR
Calcium Chloride	CaCl ₂	1mM	1mL	Sigma
Sodium Bicarbonate	NaHCO ₃	25 mM	2.1 g	Sigma
ddH ₂ O			1 L	

Table 2. 7 Krebs buffer recipe

All reagents were prepared in Krebs solution (Table 2.7) at the following concentrations: CGP 300nM, ISO 10nM, NHK 10μM, adrenaline 1μM, PTX 1.5μg/ml. Cells were perfused with the solutions at 37°C at a rate of 2ml/min. Initial Krebs perfusion allowed a stable baseline beating rate to be obtained and to which treatment responses were normalised to. Protocols established to assess the contractile response of β_2 AR stimulation are further described in Section 3.2.5.1.

2.8 SICM

2.8.1 System setup

Scanning ion conductance microscopy (SICM) is a non-contact method for cell topography imaging of live cells. The basic set-up of SICM, as shown in Fig 2.12. consists of a scanning probe head, a microscope interface (generally electronic hardware and computer) and a software control system to store and analyse scanned images. This is a powerful technique which allows 3D images of the surface of living cells to be produced and has a resolution capability of $<20\text{nm}$. In theory, it is based on the principle of ions flowing through a glass pipette filled with electrolytes and an electrode connected to a feedback mechanism which receives information from the ground electrode placed in the bath. The flow of ions through the pipette decreases as the pipette approaches the surface of the sample (Bhargava et al., 2013; Hansma et al., 1989; Korchev et al., 1997).

SICM relies on the increase of resistance due to the decrease of ionic flow in the electrolyte-containing micropipette when it approaches the non-conductive surface of the cell. The ion current flowing through the pipette is measured and fed back to the piezo-drive that moves the pipette upwards to keep the pipette-surface distance constant. The use of an ion-current feedback mechanism ensures that the distance between the pipette tip and the sample can be maintained, as there is a drop in current when the pipette approaches the cell surface. At the same time the micropipette movement is recorded while the pipette scans the surface of the cell, and that provides a topographical image of the cell surface (Bhargava et al., 2013; Korchev et al., 1997).

The SICM system was composed of the conventional inverted microscope (Diaphot 200, Nikon Corporation, Tokyo, Japan), a high-performance piezo actuator (PI, Germany) and the ICnano sample scan system (Ionscope Ltd, UK), controlled by software (ScanIC 1.9.3.248 version). The scan head of the ICnano system consisted of a three axis piezo-translation system (Physik Instrumente, UK) with a $100 \times 100 \mu\text{m}$ x-y piezo-stage for sample positioning and $38 \mu\text{m}$ z-axis piezo-actuator for the vertical

movement of the pipette, mounted on the microscope stage. The SICM components were placed on an anti-vibration table shielded by a Faraday cage to protect it from electromagnetic fields (fig 2.12).

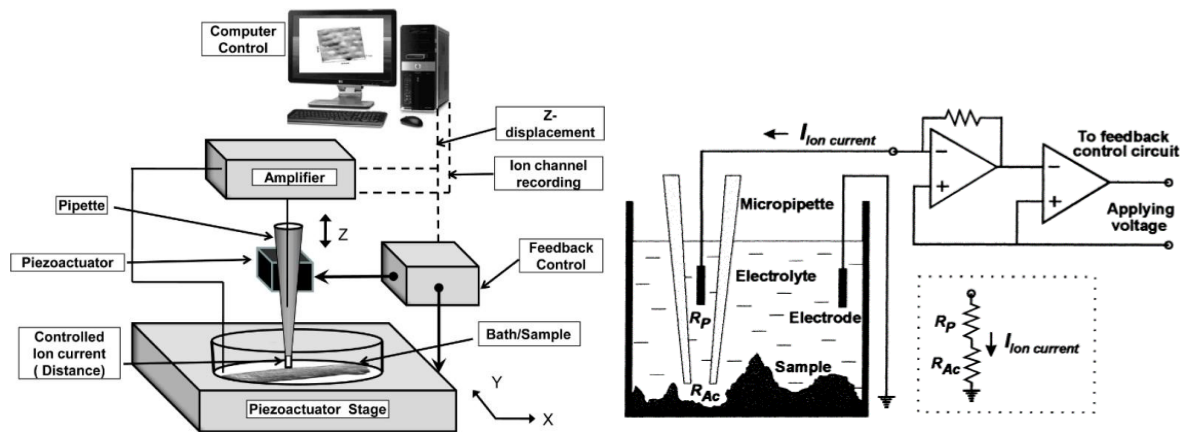


Figure 2. 12 Diagram depicting the SICM system set-up

Its major components: an ion current sensitive glass micropipette probe, a piezoelectric translation stage, the feedback generator and a computer interface to the microscope. The electric circuit of the stage showing the sample holder filled with an electrolyte. The proximity of the micropipette strongly influences the resistance and thus alters the ion current flowing through the pipette. This plays as a feedback mechanism which controls the vertical position of the micropipette (Bhargava et al., 2013; Korchev et al., 1997).

2.8.2 Reagents and protocols

hESC-CMs were plated at the centre of glass-bottom MatTek dishes at D30 of differentiation at low density to achieve a single cell culture. They were incubated for 3-4 days to allow for recovery prior to experimental use. At D33-34, the culture media was removed and replaced with bath/external solution (Table 4.2) and then placed on the microscope stage. The nanopipettes were prepared by pulling as described in Section 4.2, and were filled with the internal solution (Table 4.3) and then fixed to the holder. The scanning parameters were input into the software, including the pre-scan hop amplitude, fall rate, set point and resolution. Once this was set up, the initial, pre-scan was begun. The pipette was initially lowered into the bath/dish manually and then automated as it approached the sample, then the scanning process was begun.

2.9 Super-Resolution Scanning Patch-Clamp

The patch clamp technique allows the study of ionic currents in living cells using the “voltage clamp” technique. A glass pipette containing electrolyte solutions is tightly sealed onto the cell membrane facilitated by slight suction, indicated by an increase in membrane resistance (referred to as 1GΩ seal), therefore electrically isolating a single patch of the cell membrane. Currents fluxing through any channels in the isolated patch region flow through the glass pipette and are recorded by an electrode connected to an amplifier. This is the cell-attached patch clamp recording method. The resulting current which flows through the electrode is assumed to flow exclusively along the cell membrane. The patch clamp technique allows single ion channels or receptor properties under physiological conditions as well as under drug administration to be studied.

2.9.1 Super-resolution patch-clamp technique

Super-resolution patch-clamp technique is an advancement on the patch-clamp technique as it allows the combination of scanning ion conductance microscopy (SICM) using enhanced nanoscale imaging with the conventional cell-attached patch-clamp. This allows topographical images to be recorded and then simultaneous recordings of ion channels to be made by selecting a defined region. Previous attempts to combine both techniques faced some challenges. Firstly, they were limited by the need for a fine-tipped pipette (<100nm internal diameter) for scanning at high spatial resolutions in order to resolve nano-structures on the cell surface. Secondly, the same pipette used for scanning needed to have a diameter large enough to increase the probability of capturing functional ion channels within the patch region. The limitation here was that, a pipette with a large diameter for capturing embedded ion channels could not reveal topographical structures with high enough resolution. Therefore, an advancement to the original method was developed where computer-controlled piezo was used to

progressively clip the tip of the pipette after the topography scan (~100nm internal diameter) to obtain a wider, desired tip diameter for the patch (~300 nm internal diameter) (Bhargava et al., 2013).

2.9.2 Super-resolution scanning patch-clamp system set-up

The system setup was built with components of the SICM system and Axopatch 200A patch-clamp amplifier (Molecular Devices, USA) and was used to record ion channel activity. The cell-attached currents were digitized using Digidata 1200B and a pClamp 10 data acquisition system (Axon Instruments; Molecular devices).

2.9.3 Pipette clipping method

After generating the SICM topographical image, the pipette was clipped for LTCC recordings in the following way. The pipette was moved to a region of the cell culture dish away from any cells or debris. The piezo-controlled clipping mode was selected, and a 10-fold increase in fall rate was applied (from 50-60 nm/ms to 300-700nm/ms) to dramatically drop the pipette towards the base of the culture dish. This would cause the pipette to crash into the culture dish and slightly break the tip of the pipette, thus increasing its diameter. This is observed as a drop in resistance (Salick et al., 2014). After clipping, the fall rate was returned to 50-60nm/ms. Generally, iPSC-CMs have a flat, unstructured surface and do not display T-tubular membrane domains, pipettes with a lower resistance of 16-20 Ω M, hence larger tip diameter, was sufficient. After clipping the tip, by clicking on the chosen area of the scanned cell image, the pipette was moved back to the region of interest using the piezo and SICM software. The “non-contact mode” of SICM was switched off and the pipette would be gently lowered towards

the cell until it was close enough to touch the cells membrane. Upon reaching the membrane of the cells, the electrode resistance would increase significantly by 10-15%, indicating the patch clamp was ready to be performed. At this point, gentle negative pressure (suction) was applied through the pipette using a syringe to form a tight seal between the pipette and the cell membrane. A successful seal was indicated by a high resistance in the range of $>1\text{G}\Omega$. This configuration is referred to as cell-attached. Activity of the LTCC within the patch region was recorded (fig 2.13).

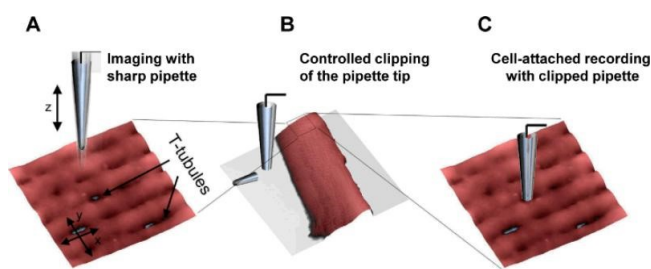


Figure 2. 13 Nanopipette clipping procedure for patch-clamp

A) High resistance pipette is used to resolve the topological structure of the cardiomyocytes. B) The pipetter is then moved to a region within the dish which is clear of cells and debris. The fall rate is increased to clip the tip of the pipette. C) the pipette is moved back to the area of interest on the cell and patch-clamp is performed (Bhargava et al., 2013).

2.10 STATISTICAL ANALYSIS

Analysis of each technique used is described in each chapter with details of how all data was recorded and analysed, as well as the statistical tests conducted to test for statistical significance. A brief description is given below.

Statistical analysis for all data was performed on GraphPad Prism 5. The statistical significance was set at $p < 0.05$ and data has been reported with $\pm$ SEM (standard error of the mean) unless otherwise stated. The number of replicates, i.e. hPSC-CM differentiation batches are indicated by 'n' as described in the figure legends and text. All data was tested for normality using Komogorov-Smirnov test. If the data passed the normality test (i.e. the data has a Gaussian distribution) then parametric tests were conducted. If the data did not have a Gaussian distribution, then non-parametric tests were conducted. P-values of $p \leq 0.05$ (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***) were considered significant.

The statistical tests conducted to analyse data obtained from experimental work were the Students T-test or its equivalent Mann-Whitney U-test was conducted when comparing two categories. The One-way Analysis Of Variance (ANOVA) with the Bonferonni correct post-hoc tests or its non-parametric equivalent Kruskal-Wallis ANOVA with Dunns correction were conducted when comparing more than 2 categories of data. The F-statistic was used on the dose response curves to find out if the EC_{50} between the two populations were significantly different.

CHAPTER 3: INVESTIGATING THE ROLE OF β_2 AR HAPLOTYPES ON CONTRACTILE ACTIVITY IN iPSC-CMs

3.1 INTRODUCTION

The activity and regulation of the β ARs will be investigated in several ways throughout this thesis, with a primary focus on the β_2 ARs. To begin with, the functional activity of the 4 common β_2 ARs haplotypes will be explored in this chapter.

The β ARs are the primary targets for the endogenous catecholamine hormone epinephrine and the neurotransmitter norepinephrine, as well as exogenous agonists and antagonists. They are responsible for mediating physiological responses to environmental cues and play a major role in the pathophysiology of human heart disease (Wang et al., 2018). The activation of these receptors promotes coupling to the G-stimulatory (G_s) subunit of G-proteins which increases adenylyl cyclase (AC) activity and elevates cAMP levels, leading to PKA activation (Gilman, 1984). Interestingly, unlike β_1 ARs, β_2 ARs can also interact with G-inhibitory (G_i) subunits, thus activating an opposing signalling pathway, dampening AC activity and decreasing intracellular cAMP (Pierce et al., 2002). This alternative coupling is considered to be cardioprotective as it allows β_2 ARs to counteract the apoptotic pathway mediated by β_1 ARs after prolonged stimulation, thus promoting cell survival (Johnson & Terra, 2002).

There are multiple genetic variants of the β_2 ARs which exist in the population (Johnson & Terra, 2002). The two most common functionally relevant single nucleotide polymorphisms (SNPs) are in codon 16 (Gly to Arg; G to R) and 27 (Glu to Gln; E to Q) at the N-terminal of the receptor (Ahles & Engelhardt, 2009; Danielewicz, 2014; Ferreira-Santos et al., 2017). They have been associated with a complex array of phenotypes as well as increasing susceptibility to various diseases, including hypertension, heart failure, asthma and cystic fibrosis (Iwamoto et al., 2011; Maxwell et al., 2005; Munakata et al., 2006). Although these SNPs are not thought to directly cause disease, they are considered to be important risk factors or modifiers of response to stress and drugs, thus influencing disease outcome.

The way in which these polymorphisms modulate β_2 AR response is not fully understood, however, there are indications of effecting agonist-mediated desensitisation and receptor down regulation (Green et al., 1994), receptor trafficking and phosphorylation (Gough, 2011; Koryakina et al., 2012); though the results are quite controversial. Currently, we are limited by the variability in the background genotype of model systems available which is known to influence phenotype (Chruscinski et al., 1999; Kondrashov et al., 2018). To overcome this, Alexander Kondrashov and colleagues in Professor Chris Denning's lab at Nottingham University, engineered an isogenic set of hESC-CM cell lines where only the specific SNPs of interest are changed to produce either of the 4 haplotypes and the remainder of the genetic background remains unaltered. As described in Section 2.1.5.1, a two-step approach combining CRISPR/Cas9 and the *PiggyBac* transposon technique was used. This allowed the activity of the β_2 AR haplotypes with respect to the specific polymorphic modification to be studied, without interference from the remainder of the genetic background.

In this chapter, we aimed to investigate the influence of the four β_2 AR haplotypes (GE, GQ, RE, RQ) on the cAMP handling of the cell through measuring real-time changes to intracellular cAMP upon stimulation of the β_2 AR with isoprenaline. Secondly, the functional activity of the receptor haplotypes were assessed through measuring the chronotropic response of hESC-CMs upon β_2 AR stimulation. β_2 AR interaction with G_i was investigated to study the influence of the haplotypes on the level of biased agonism. In order to investigate G_i activity, hESC-CMs were treated with PTX as an inhibitor of G_i function and the change in beating rate in response to β_2 AR stimulation was measured. β_2 AR- G_i interaction was also investigated in response to chronic stress mediated by pre-treatment with adrenaline as described in previous literature.

3.2 METHODS

3.2.1 Cardiac differentiation of the genetically modified hESC

Human embryonic stem cells (hESCs) of the HUES7 cell line were used to produce genetically modified cell lines which stably expressed one of the four β_2 AR haplotypes encoding the amino acid arginine or glycine at position 16 and either glutamine or glutamic acid at the amino acid position 27. The CRISPR/Cas9 system was utilised to produce these haplotypes by Alexander Kondrashov in Chris Dennings lab, Nottingham University. These hESCs were then prepared for cardiac differentiation as described in section 2.1.5.2 (Fig 3.1).

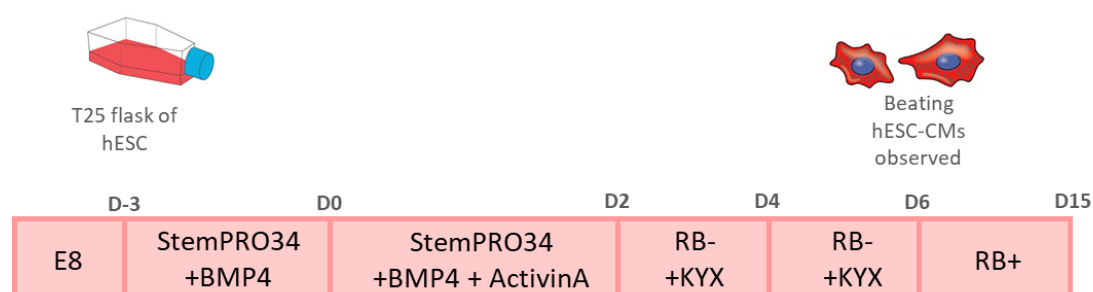


Figure 3. 1 hESC differentiation protocol

3.2.2 hESC-CMs maintenance and dissociation

hESC-CMS were cultured on plastic T25 culture flasks in RB+ medium. A D15 cells were dissociated and re-plated as the differentiation process involves killing off non-myocytes, and this leaves gaps in the culture. The dissociation was conducted as described in Section 2.1.5.3.

For FRET and contraction studies in this chapter, 70 μ L of the cell suspension were plated on the centre of the pre-prepared, Vitronectin-coated (1h at 37°C) MatTek dishes and incubated at 37°C for at least 2 hours to allow cells to adhere to the dish. After these 2 hours, 1.5mL of RB+ was added to the culture dishes for at least 3 days before any experiments were conducted in order to allow cells recovery. Cells were used at D37 $\pm$ 3 days post initiation of cardiac differentiation.

For FRET studies, cells were prepared at low densities to obtain single-cell cultures. For contraction studies, cells were prepared at high densities to obtain monolayers as this promotes cell contractility.

3.2.3 hESC-CMs transfection with FRET biosensor

For FRET experiments, hESC-CMs were plated on glass-bottomed MatTek dishes at D30-32 of differentiation and maintained in RB+ for a further 3-5 days to allow cell recovery. Cells were seeded at low densities to obtain single cell cultures. At around D35 cells were transfected with the pTEV FRET sensor using JetPRIME (Polyplus) transfection kit following manufacturer's protocol as described in Section 2.7.7 (Fig 3.2). Generally, a 20-30% transfection efficiency was obtained. Cells expressing the sensor evenly throughout the cytosol were selected for experimentation. No significant toxicity was observed upon visualisation under a microscope.

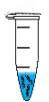
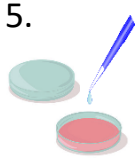
1.  Combine 200ng pTEV DNA + 50µL JetPrime Buffer
2.  Vortex on low speed for 10-15 seconds
3.  + 2µL JetPrime Reagent and vortex for 15 seconds
4.  Incubate for 10 minutes at room temperature
5.  Add transfection mixture to cells and incubate for 24-72 hours

Figure 3. 2 pTEV FRET sensor transfection protocol of hESC-CMs with jetPRIME

3.2.4 β_2 AR-ISO dose response curves on hESC-CMs haplotypes by FRET

3.2.4.1 FRET reagents and protocols

Dose response of β_2 AR stimulation with ISO was conducted to assess the EC_{50} levels of the four receptor haplotypes. hESC-CMs MatTek dishes were firstly removed from the incubator and media was aspirated. 0.5mL of warmed FRET buffer was used to wash the dish, then 500 μ L of CGP20712A (CGP) (Sigma Aldrich, Poole UK) (100nM) prepared in FRET buffer was added. Cells were incubated in CGP for 10 minutes before starting the FRET recording. After CGP incubation, β_2 AR was stimulated with 500 μ L 0.1nM ISO. Once a plateau was obtained, 500 μ L of ISO was added for the final concentration of 0.3nM. Again, when a plateau was obtained the next ISO solution was added. The final ISO concentrations ranging from 0.1nM to 3nM or 3nM to 100nM were prepared in FRET buffer and were added in order starting from the lowest concentration. At the end of each experiment, NKH at the final concentration of 10 μ M was added to obtain a maximal cAMP output response to which each ISO was normalised (Fig 3.3).

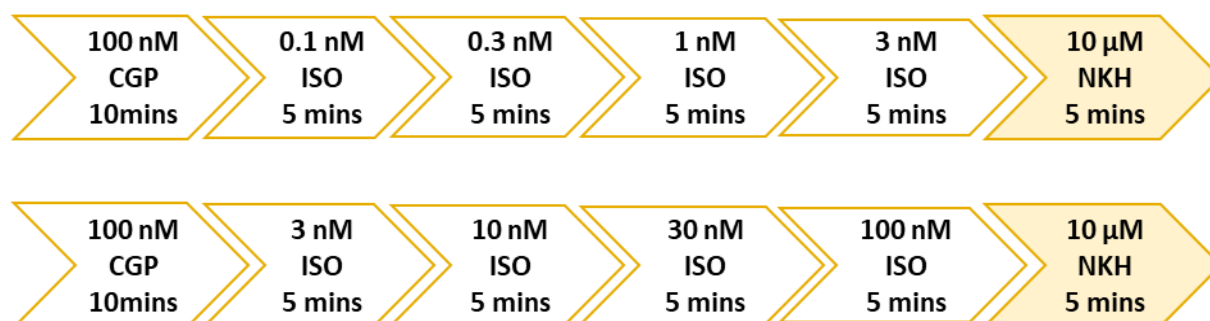


Figure 3. 3 Protocol for dose responses of β_2 AR to isoprenaline used for FRET stating final concentrations of solutions in the bath

3.2.4.2 FRET analysis

Analysis of the FRET results were conducted in Microsoft Excel. The ratio between the fluorescence recorded from the YFP channel and the CFP channel was calculated and plotted to obtain a regular YFP/CFP FRET trace. These results are shown as line graphs with the “FRET ratio (Venus/Turquoise)”

plotted (Fig 3.4). From this the percentage change in the ratio from baseline to the individual ISO treatment in question was calculated and normalised to the NKH response, and is shown as “% change in FRET ratio”. The final dose response trace is presented as a normalised curve. From this curve, the EC50 was reported. Statistical significance between the 4 haplotypes was tested using the F-statistic in GraphPad Prism 5.

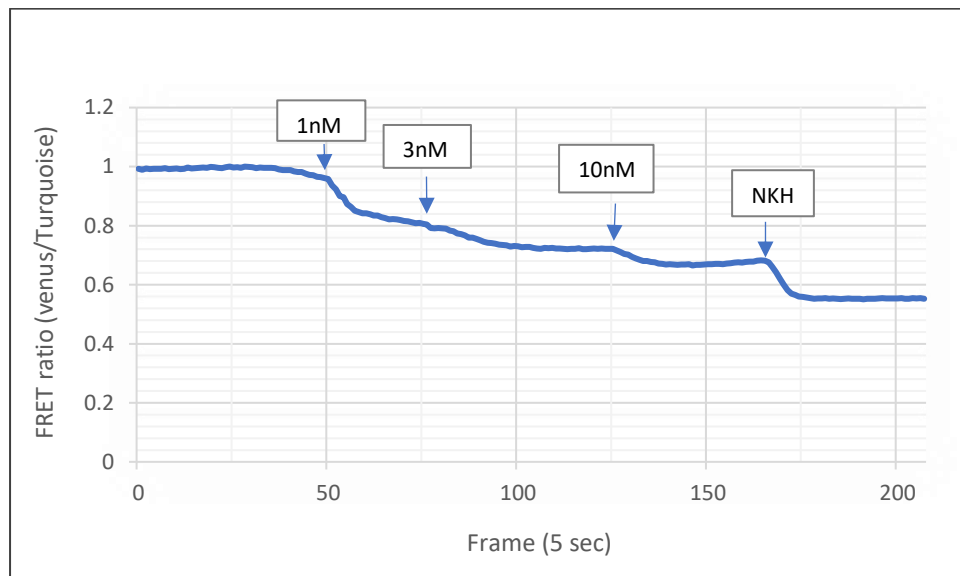


Figure 3. 4 Example of FRET trace of dose response curve

The isoprenaline concentrations were added in sequential order starting with the lowest concentration. The subsequent concentration was only added after a plateau was reached. Each experiment was concluded by the addition of NKH to which each ISO response was normalised.

3.2.5 Contraction recordings of hESC-CMs by IonOptix

3.2.5.1 Contraction reagents and protocols

IonOptix experiments were conducted to study the differences in contractility of the hESC-CMs of the four β_2 AR haplotypes under basal stimulation under basal stimulation and upon stress induction. The hESC-CMs at D30-D35 were re-plated at high densities on glass-bottom MatTek dishes to obtain monolayers, for a minimum of 48h prior to experimentation. At the start of each experiment, media was removed and replaced by warmed Krebs solution. This was applied through a perfusion system

which oxygenated and warmed to 37°C before application onto the cells. The flow was maintained at 2mL/min (Fig 3.5).

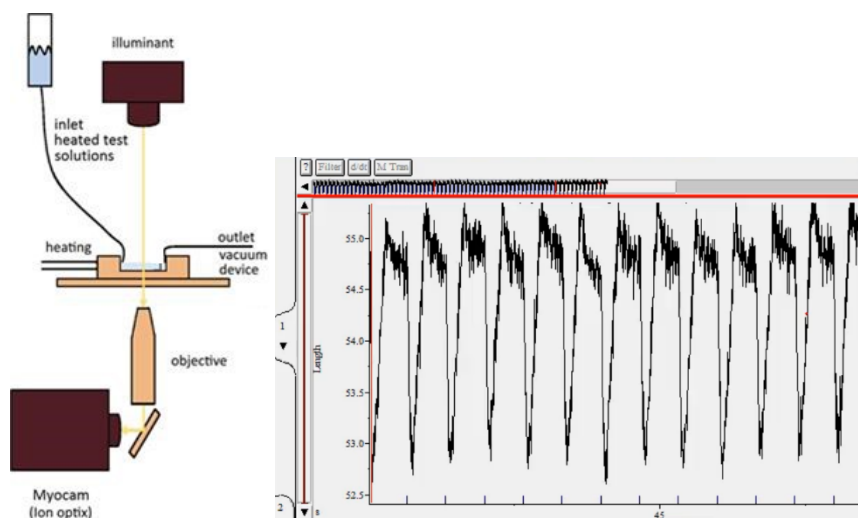


Figure 3. 5 The IonOptix system setup for recording iPSC-CMs contraction and the data output. Each peak represents one beat/contraction.

All reagents were prepared in KH solution (Table 2.6) at the following concentrations: CGP 300nM, ISO 10nM, NKH 10μM, adrenaline 1μM, PTX 1.5μg/ml. Cells were perfused with the solutions at 37°C at a rate of 2ml/min. Initial Krebs perfusion a stable baseline beating rate to which treatment responses have been normalised to. Protocols established to assess the contractile response of β_2 AR stimulation are as follows (Fig 3.6):

- A) Control: Standard β_2 AR response:
15min KH, 10min selective β_1 AR blocker CGP, followed by 20min ISO+CGP.
- B) PTX: To assess the influence of G_i proteins:
3h pre-treatment with PTX, 15min KH perfusion, 10min CGP and then 20 min ISO+CGP.
- C) ADR: In vitro stress model by pre-treating cells with adrenaline (Protocol adapted from (Kondrashov et al., 2018)):
15min KH, 20min adrenaline, then 10min CGP, then 20 min ISO+CGP.

D) ADR+PTX: To assess the role of G_i proteins on cellular contractility during induced cardiomyocyte stress (protocol adapted from (Kondrashov et al., 2018)):
pre-treatment with PTX, 15min KH, 20min adrenaline, 10min CGP and then 20min ISO+CGP.

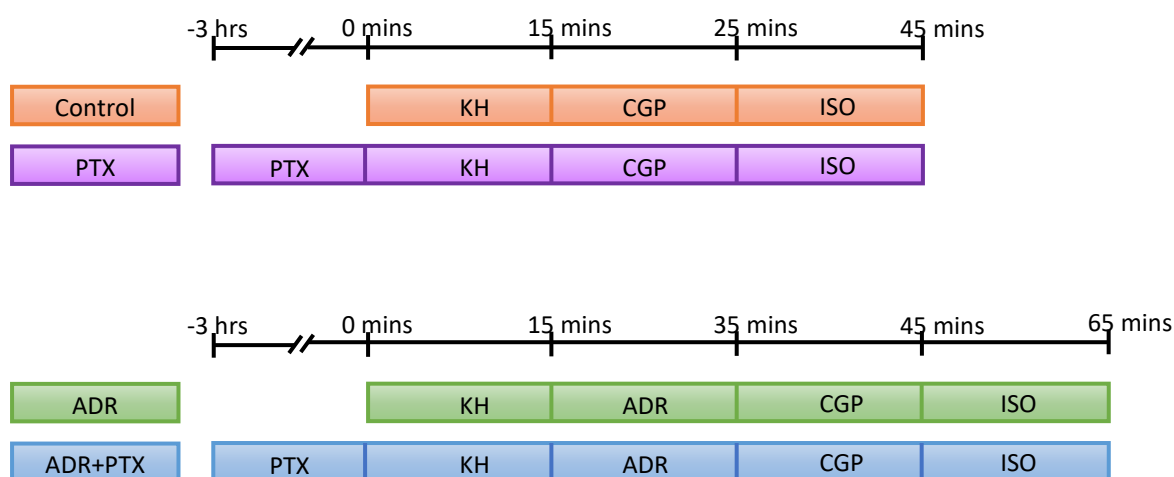


Figure 3. 6 Drug administration protocol during IonOptix experiments

3.2.5.2 IonOptix Analysis

Analysis of contraction data from the IonOptix system were conducted manually. Contraction recordings were taken during a stable period of the KH treatment, at each drug addition and also at 5-minute intervals during the ISO treatment (Fig 3.7). The experiment was concluded after a 20-minute period of ISO treatment. From the data output, the number of peaks represent the number of beats and were recorded manually by counting the number of peaks over a 60-second time period. In all experiments, the baseline recordings were taken during a stable period of the recording during KH perfusion. For each drug treatment, the response (measured by bpm) was taken during the last 60-seconds of the drug treatment and regularly at 5-minute intervals over the course of the 20-minute ISO stimulation. The bpm is shown as either “absolute bpm” values or as “% relative to baseline” (normalised to KH). Statistical significance was tested through Student’s T-test and One-Way ANOVA.

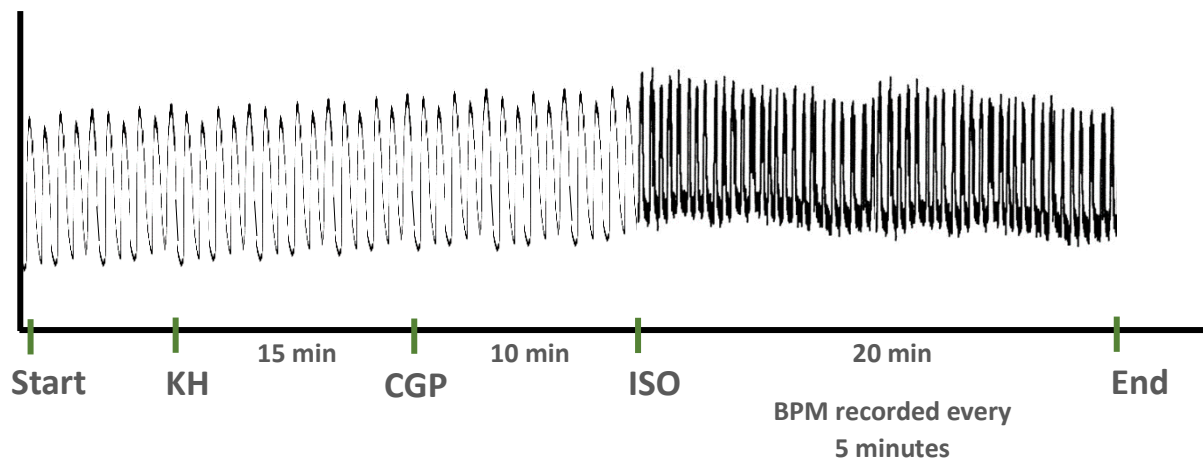


Figure 3. 7 Example of IonOptix recording

Illustration recording of an IonOptix recording example with the respective treatments highlighted along with the duration of the treatment. Each peak is the result of movement of the tracked region of the cell and represents a single contraction. After treatment with ISO, it was expected that the beating rate would increase, which can be seen as an increase in the number of peaks/beats per minute.

3.2.6 Statistical analysis

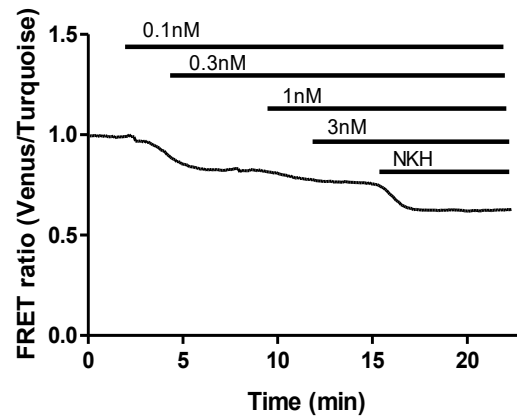
The statistical tests conducted has been described in each experimental method section. Generally, the statistical analysis used to analyse data obtained for experimental work was Students T-Test, which were performed using GraphPad Prism 5. The statistical significance was set at $p < 0.05$ and data has been reported with $\pm$ SEM (Standard error of the mean).

3.3 RESULTS

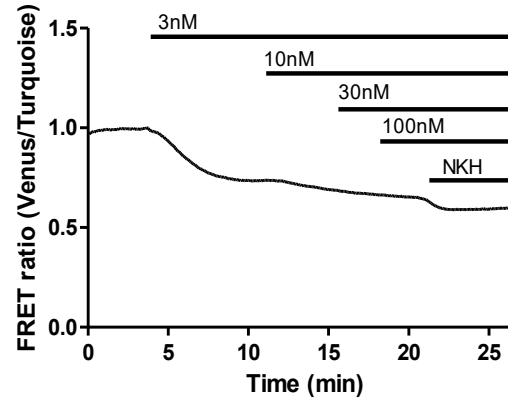
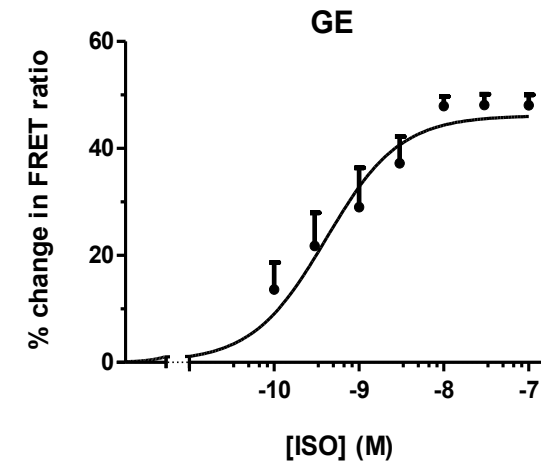
3.3.1 Haplotype-dependent sensitivity of β_2 AR to isoprenaline on cAMP activity

Firstly, the sensitivity of the GE, GQ, RE, and RQ β_2 AR haplotypes was explored. β_2 ARs were stimulated with isoprenaline concentrations ranging from 0.1-100nM and cAMP output was measured using FRET. Concentration-response curves for each haplotype were produced based on the change in FRET ratio detected at each concentration of isoprenaline in the presence of CGP for β_2 AR-specific stimulation. The cAMP output was normalised to NKH, an AC activator for maximal cAMP release. The GQ and RQ haplotypes showed relatively little response to receptor stimulation with 0.1nM to 3nM isoprenaline concentrations in comparison to GE and RE variants which showed gradual increases in FRET response with isoprenaline concentration increments. The GE haplotype had obtained its maximal response by 3nM isoprenaline and RE haplotype achieved a maximal response by 30nM. Between 3nM and 100nM isoprenaline concentrations, GQ, RE and RQ continued to increase gradually in cAMP output. The GQ and RQ haplotypes continued to be responsive until 100nM isoprenaline. From these FRET experiments, a concentration-response curve was generated and the half-maximal effective concentration (EC_{50}) was calculated. GE showed the greatest sensitivity to β_2 AR stimulation with an EC_{50} of 1nM 95% CI [0.1, 12.3]. RE, RQ and GQ are significantly less sensitive than GE with an EC_{50} of 4nM [1.8, 5.4], 48nM [6.4, 153.3] and 28nM [20.9, 38.9] respectively ($p<0.05$) (Fig 3.8). There was no significant difference in maximum FRET response between haplotypes.

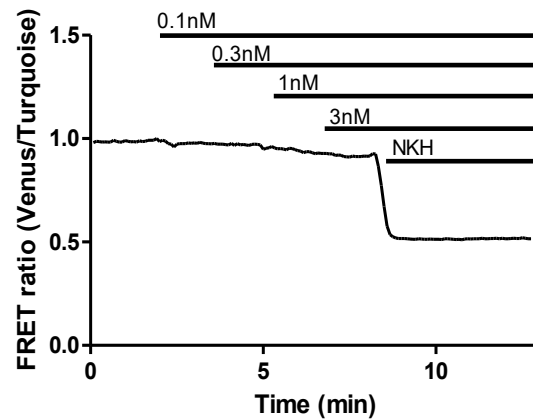
**Representative FRET Trace
GE (0.1-3nM ISO)**



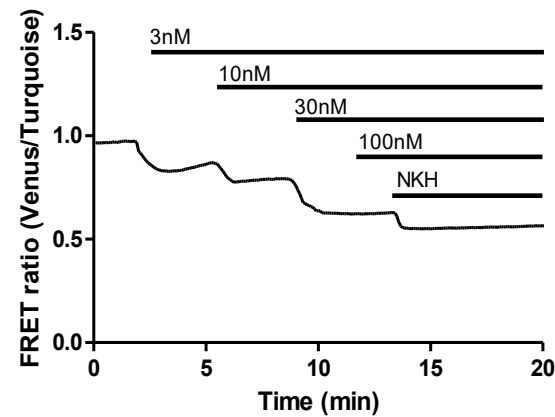
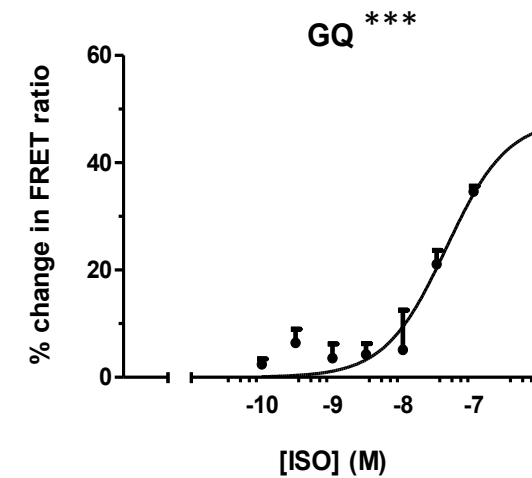
**Representative FRET Trace
GE (3-100nM ISO)**



**Representative FRET Trace
GQ (0.1-3nM ISO)**



**Representative FRET Trace
GQ (3-100nM ISO)**



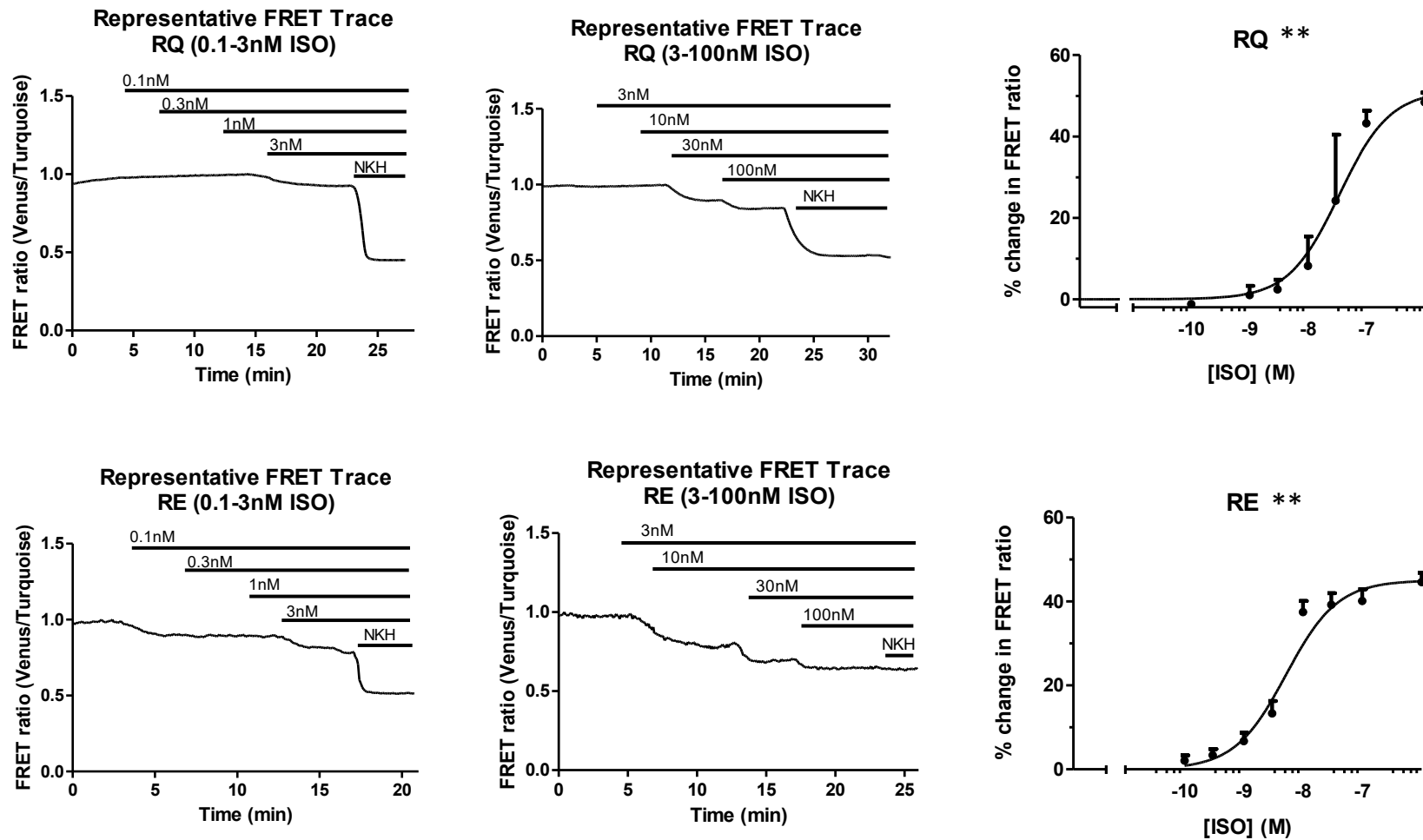


Figure 3. 8 Representative FRET traces and concentration response curves of β_2 AR haplotypes

Concentration-response curves measuring β_2 AR-cAMP production upon isoprenaline (ISO) stimulation in the presence of β_1 AR-blocker CGP20172A by FRET in hESC-CM for each haplotype. N values GE (4,2 cells,preparations); GQ (4,4); RQ (3,2); RE (9,4). Significantly different from GE, *** $P < 0.001$, F-test.

3.3.2 Haplotype-dependant sensitivity of β_2 AR to isoprenaline on cell contraction

Further to the FRET studies, the functional activity of β_2 AR was also measured through recording contractile output. Cardiomyocyte beating rate at 5-minute intervals after β_2 AR stimulation with 300nM CGP + 10nM ISO over a 20-minute period was recorded from a region where a steady beating rate was obtained. The absolute beating rate and the normalised beating rate are reported.

The beating rate in control experiments were conducted first in all four β_2 AR haplotypes; GE, GQ, RE and RQ. The baseline contractility was significantly higher at the start of the experiment in the RE haplotype with a 127 ± 5 bpm in comparison to the other three haplotypes which were between 50 to 60 bpm. Upon stimulation with ISO, all haplotypes showed an increase in beating rate after the initial 2 minutes, with GE and GQ showing the greatest increase, 115 ± 13 bpm and 93 ± 12 bpm respectively. Over the 20-minute period of stimulation with ISO, GE and GQ showed a significant drop in beating rate after this maximum, to 81 ± 8 bpm and 58 ± 4 bpm respectively ($p < 0.05$), and there was a small decrease in RQ by 20 minutes, from 91 ± 5 to 74 ± 6 bpm. RE had the greatest initial beating rate with a small increase to 143 ± 5 bpm at 2 minutes of stimulation, and 137 ± 8 bpm at 20 minutes of stimulation (Fig 3.9).

To make an accurate comparison of how the beating rate changed upon stimulation with ISO the normalised beating rate was calculated, allowing us to study the relative change in beating rate. GE showed a significantly greater percentage increase at 2 minutes of stimulation ($242 \pm 30\%$) in comparison to GQ, RQ and RE ($176 \pm 13\%$, $136 \pm 6\%$ and $115 \pm 6\%$ respectively) ($p < 0.05$). After 20 minutes of ISO stimulation a decrease in the beating rate was noted in all four haplotypes with the beating rate change dropping to $160 \pm 9\%$, $113 \pm 13\%$, $110 \pm 6\%$ and $109 \pm 4\%$ over baseline for GE, GQ, RQ and RE respectively, although the drop was only significant for the G variants ($p < 0.05$). GQ, RQ and RE haplotypes showed the beating rate return back to basal levels after 20 minutes of stimulation. However, GE remained notably higher above baseline (Fig 3.10).

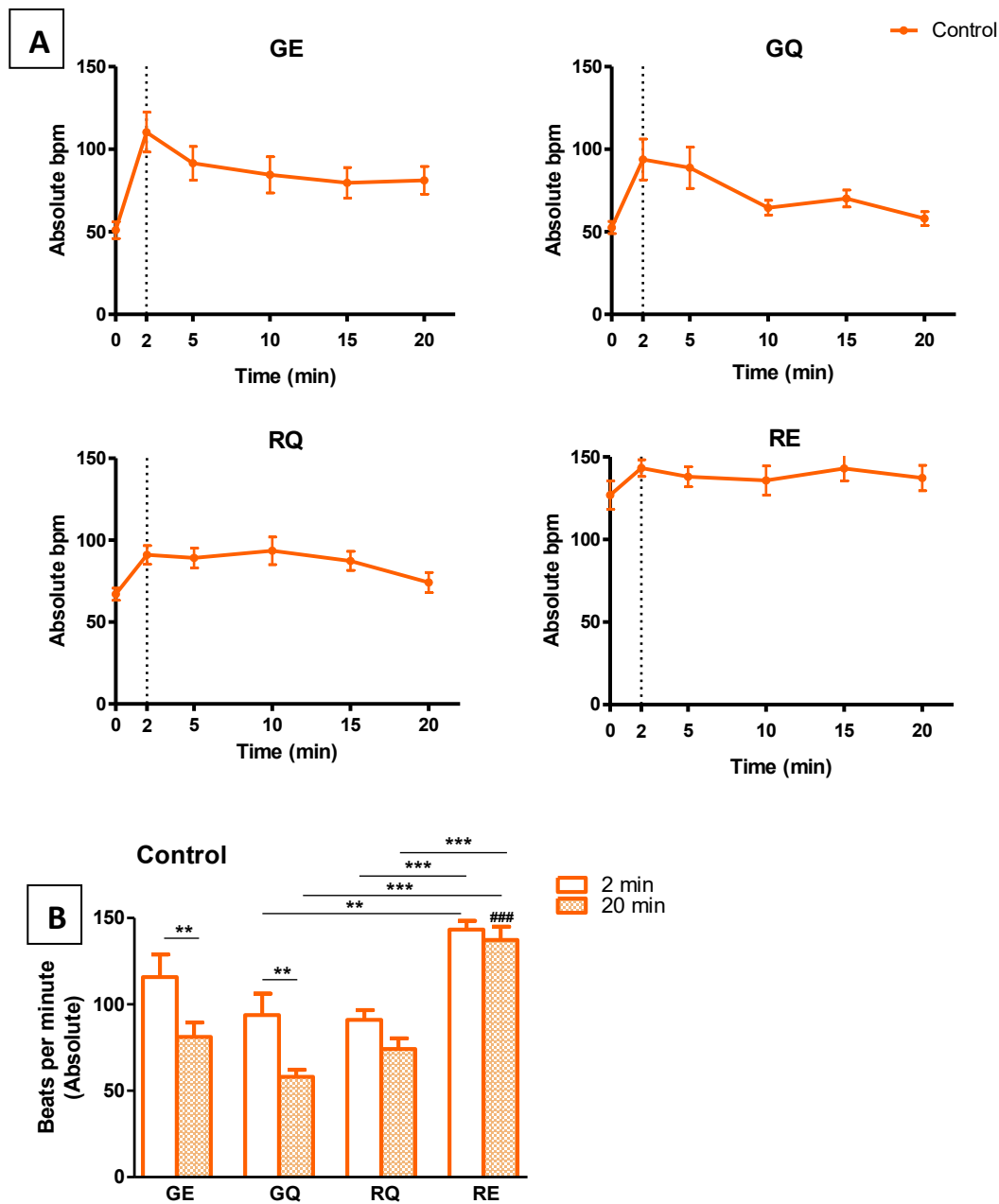


Figure 3. 9 Beating rate in β_2AR haplotypes upon stimulation with ISO

A) Beating rate of heSC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype over 20 minutes of β_2AR stimulation with ISO in the presence of ICI. B) Bar graph showing the beating rate of heSC-CMs with these 4 haplotypes at 2mins and 20mins through ISO stimulation. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n/N)=cells/batches.

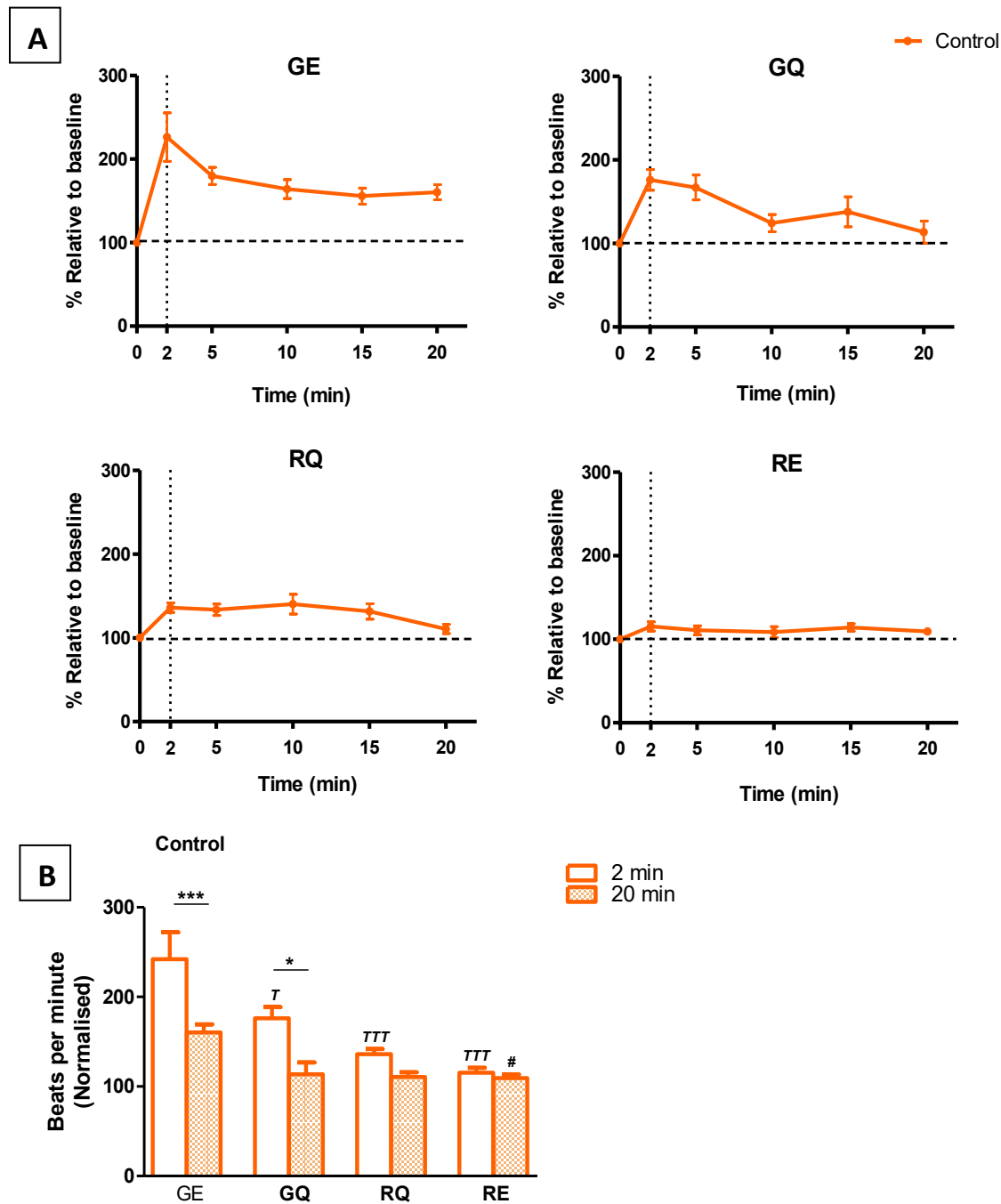


Figure 3. 10 Normalised beating rate in β_2AR haplotypes upon stimulation with ISO

A) Beating rate of heSC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype normalised to basal contractility over 20 minutes of β_2AR stimulation with ISO in the presence of ICI. B) Bar graph showing the beating rate of heSC-CMs with these 4 haplotypes at 2mins and 20mins through ISO stimulation normalised to baseline. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches.

3.3.3 Investigating the influence of β_2 AR haplotype on G_i effect

Next, we wanted to understand whether the desensitisation observed is due to an underlying protective G_i effect being activated with β_2 AR stimulation which is the cause for the progressive decrease in beating rate. PTX was used to inhibit the role of G_i throughout β_2 AR stimulation with ISO.

In PTX treated cells, all four haplotypes showed an initial increase in beating rate at 2min of ISO stimulation. The RE haplotype, again, had the highest initial basal beating rate of 98 ± 6 bpm in comparison to GE, GQ and RQ which were 50 ± 3 , 44 ± 4 and 82 ± 4 bpm. The R haplotype seems to display the higher initial/basal beating rates. At the 2-minute time point of ISO stimulation, the absolute beating rate of all four haplotypes, GE, GQ, RQ and RE increased to 92 ± 5 , 62 ± 2 , 110 ± 7 and 115 ± 21 bpm respectively. GE showed the largest increase in beating rate from Time:0, whereas the other haplotypes showed modest increases. Interestingly, over the 20 minutes of stimulation, the beating rate did not return back to baseline in all four haplotypes as was seen in the control experiment. In the RQ control experiment, there is a relative decrease in beating rate over the 20 minutes of ISO stimulation which would be due to receptor desensitisation. By blocking G_i with PTX treatment, this drop in beating rate seems to be prevented. With the GQ, GE and the RE haplotypes, PTX treatment seems to prevent the increase in contraction altogether, preventing maximal contraction rate being achieved (Fig 3.11).

After normalisation, PTX-treated GE had the highest beating rate of $187\% \pm 13$ bpm at 2 minutes of ISO stimulation; statistically greater than GQ, RQ and RE; $144\% \pm 12$, $133\% \pm 6$ and $125\% \pm 7$ respectively. In comparison to control, inhibition of G_i did not affect beating rate in any of the haplotypes as significance was not observed. After 20 minutes of stimulation the GE haplotype shows a slightly lowered beating rate of $167\% \pm 16$ bpm, whereas the others showed no change in beating rate; $147\% \pm 8$, $130\% \pm 3$ and $128\% \pm 8$ bpm of GQ, RQ and RE respectively. In comparison to control, there were no significant differences detected in contractility after G_i inhibition (Fig 3.12); indicating relatively little protective effect through β_2 AR- G_i coupling upon receptor activation.

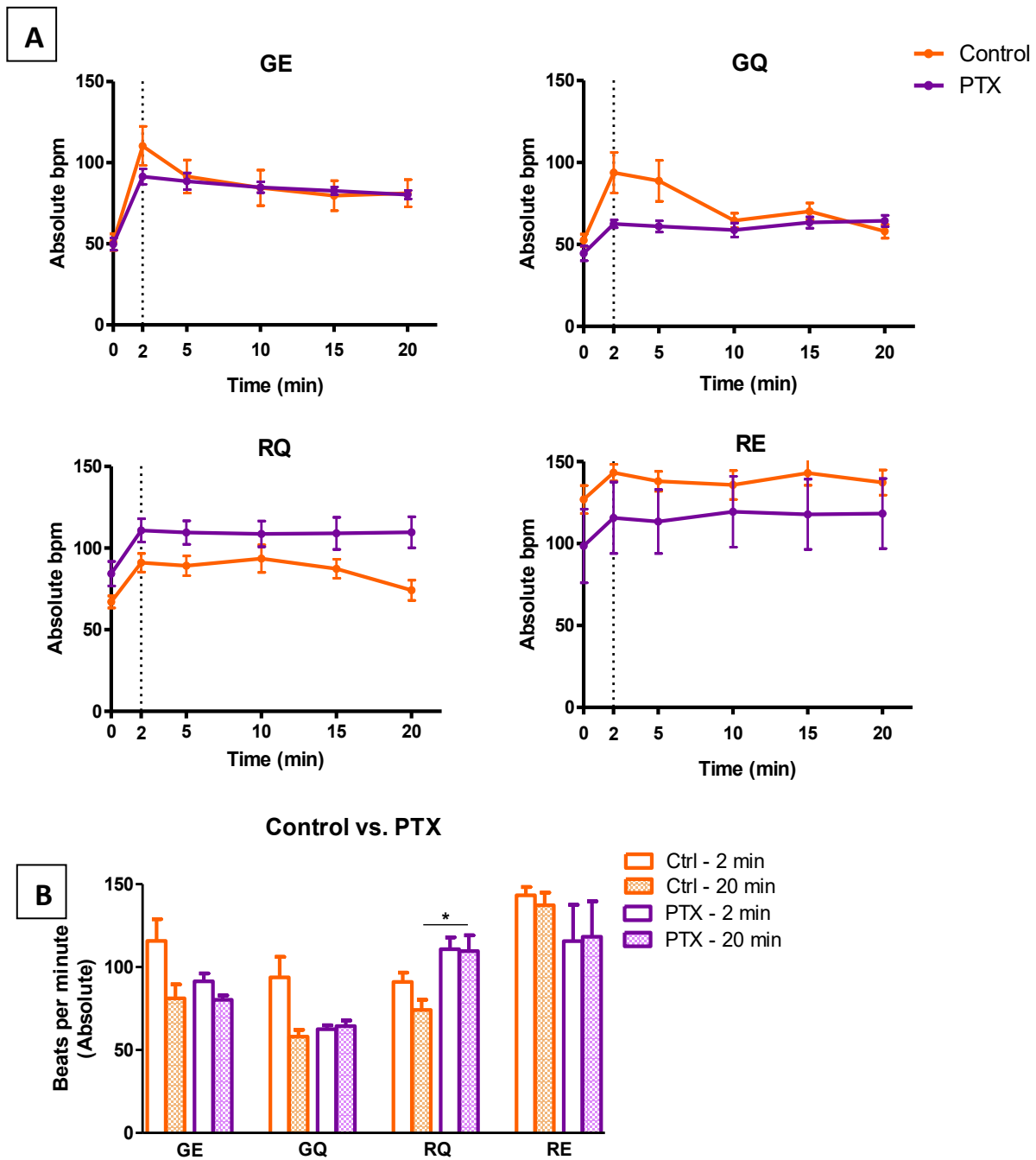


Figure 3. 11 Beating rate in β_2AR haplotypes upon stimulation with ISO (orange) with PTX (purple).

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype over 20 minutes of β_2AR stimulation with ISO in the presence of ICI (orange) with hESC-CMs GE (7/4), GQ (5/4), RQ (6/3) and RE (7/3) haplotypes pre-treated with PTX (purple). B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with PTX at 2mins and 20mins through ISO stimulation. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches.

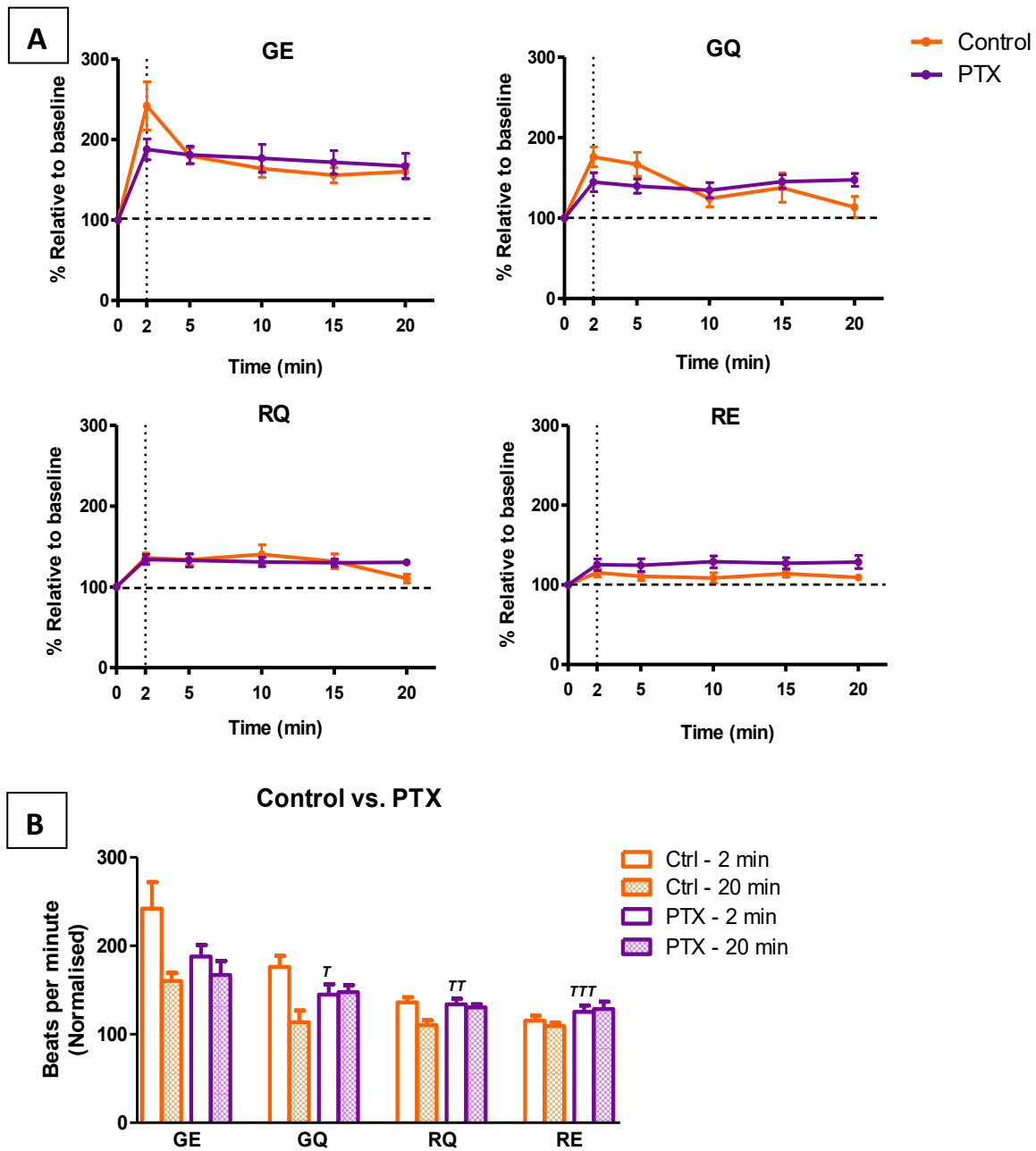


Figure 3. 12 Normalised beating rate in β_2 AR haplotypes upon stimulation with ISO (orange) and PTX (purple)

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype normalised to basal contractility over 20 minutes of β_2 AR stimulation with ISO in the presence of ICI (orange) with hESC-CMs GE (7/4), GQ (5/4), RQ (6/3) and RE (7/3) haplotypes pre-treated with PTX (purple). B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with PTX at 2mins and 20mins through ISO stimulation normalised to baseline. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches.

3.3.4 The role of the β_2 AR haplotypes upon stress by adrenaline pre-treatment

To assess whether the protective G_i effect can be made more prominent, we developed an *in vitro* stress model to accelerate the G_s to G_i switch by increasing phosphorylation of the β_2 AR (Paur et al., 2012). For this, cells were pre-treated with adrenaline, which was then washed out and the cells were allowed to recover baseline contraction rate prior to ISO stimulation. The contractility of each haplotype was then measured during 20 minutes of stimulation with ISO.

All four haplotypes showed a similar initial beating rate in comparison to control experiments, where the beating rate of GE, GQ, RQ and RE was 58 ± 3 bpm, 57 ± 2 bpm, 90 ± 15 bpm and 118 ± 18 bpm respectively. GE and GQ have a relatively similar beating rate and RQ and RE had a slightly raised beating rate. The initial rise at 2 minutes of stimulation which was seen in control cells was less prominent in GE and GQ, and was not observed in RQ and RE haplotypes. Over the 20 minutes of ISO stimulation, the absolute beating rate showed that all four haplotypes maintained a relatively constant beating rate; 69 ± 6 bpm, 61 ± 5 bpm, 94 ± 11 bpm, 112 ± 19 bpm for GE, GQ, RQ and RE respectively (Fig 3.13).

When the beating response was normalised, only GE had a slightly elevated beating response with a 34% rise in beating rate which gradually reduced over the 20 minutes, whereas GQ, RG and RE remained close to baseline. Overall, no significant changes in beating rate were observed in the four haplotypes with the pre-treatment of cells with adrenaline. In comparison to control experiments, we see desensitisation in all 4 haplotypes (Fig 3.14).

This may indicate underlying desensitising activity in the variants, which may explain reduced responsiveness of the β_2 ARs to ISO stimulation.

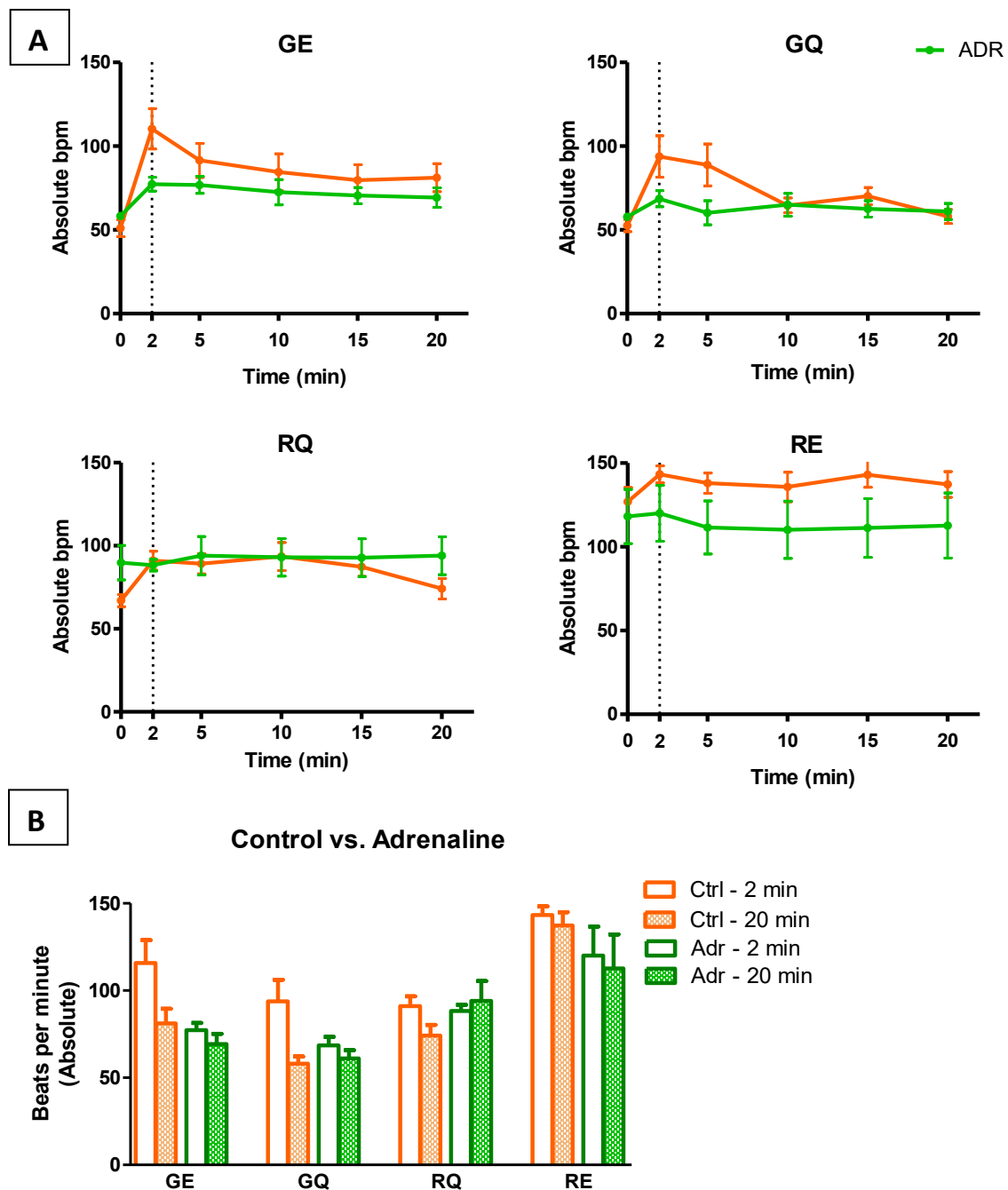


Figure 3. 13 Beating rate in β_2 AR haplotypes upon stimulation with ISO (orange) and adrenaline (green)

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype over 20 minutes of β_2 AR stimulation with ISO in the presence of ICI (orange) with hESC-CMs GE (6/4), GQ (5/3), RQ (6/4) and RE (7/4) haplotypes pre-treated with adrenaline (green). B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with adrenaline at 2mins and 20mins through ISO stimulation. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches.

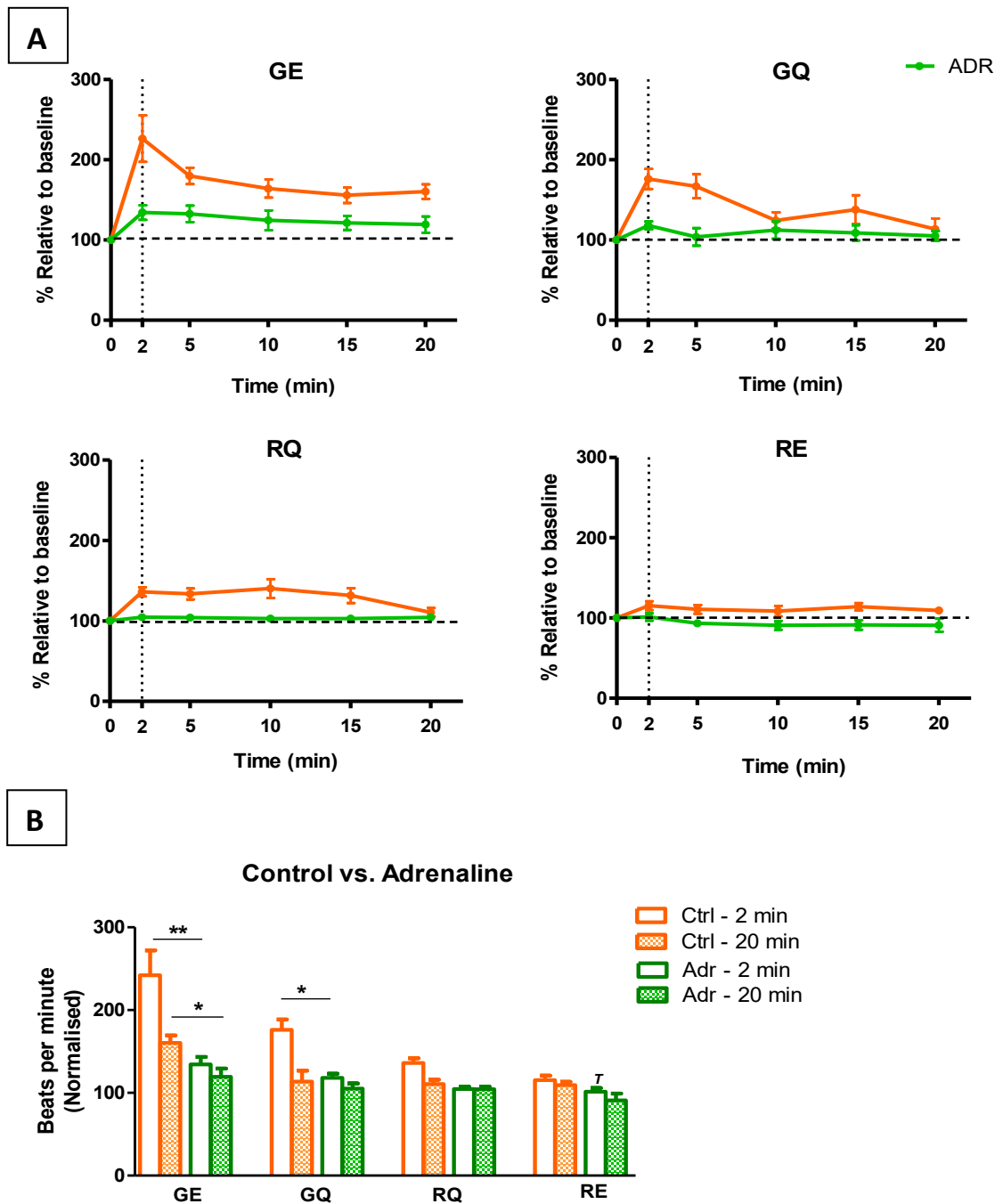


Figure 3. 14 Normalised beating rate in β_2 AR haplotypes upon stimulation with ISO and adrenaline

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/4), RQ (6/4) and RE (8/4) haplotype normalised to basal contractility over 20 minutes of β_2 AR stimulation with ISO in the presence of ICI (orange) with hESC-CMs GE (6/4), GQ (5/3), RQ (6/4) and RE (7/4) haplotypes pre-treated with adrenaline (green).

B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with adrenaline at 2mins and 20mins through ISO stimulation normalised to baseline. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches.

3.3.5 The influence of the β_2 AR haplotypes during stress with G_i inhibition

We saw a desensitisation-like effect when cells were pre-treated with adrenaline. To assess whether this was a G_i switch rather than desensitisation of the β_2 AR, we treated the cells with PTX prior to adrenaline stimulation. The adrenaline was then washed out, the cells were allowed to recover, and were then stimulated with ISO. The contractility was measure during the 20 minutes of ISO stimulation.

Upon assessing absolute beats per minute, GQ showed the lowest beating rate in both adrenaline treatment and after PTX treatment with a beating rate of 52 bpm, statistically lower than GE, RQ and RE at 2 minutes (89 ± 8 bpm, 84 ± 10 bpm, and 116 ± 15 bpm respectively) and 20 minutes (104 ± 6 bpm, 82 ± 12 bpm, and 112 ± 12 bpm respectively) of ISO stimulation. None of the haplotypes showed a change in beating rate over the 20 minutes of stimulation. Only a slight increase was seen at 2 minutes of ISO stimulation, though not significant (93 ± 5 bpm, 66 ± 3 bpm, 99 ± 11 bpm, and 124 ± 7 bpm for GE, GQ, RQ and RE respectively) (Fig 3.15).

When assessing the change in beating rate with PTX treatment by normalisation, no differences were observed between the haplotypes. Despite treatment with PTX with adrenaline, no difference in contraction rate or desensitisation was observed (Fig 3.16).

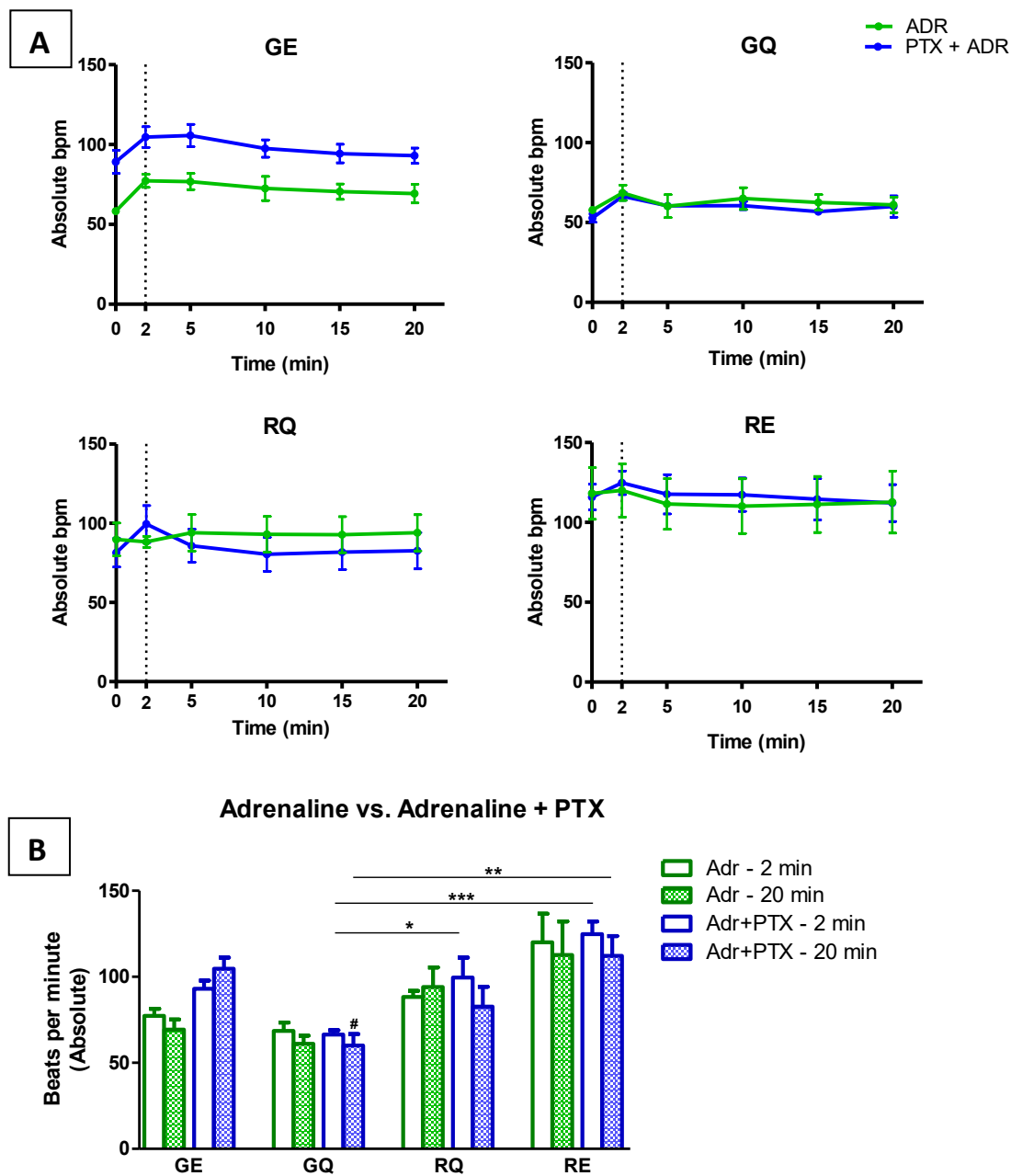


Figure 3. 15 Beating rate in β_2AR haplotypes upon stimulation with adrenaline (green) and adrenaline+PTX (blue)

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/3), RQ (7/4) and RE (6/4) haplotype over 20 minutes of β_2AR stimulation with ISO in the presence of ICI when pre-treated with adrenaline (green), and when hESC-CMs GE (6/4), GQ (5/3), RQ (7/3) and RE (6/3) haplotypes pre-treated with PTX with adrenaline (blue). B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with PTX at 2mins and 20mins through ISO stimulation. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches

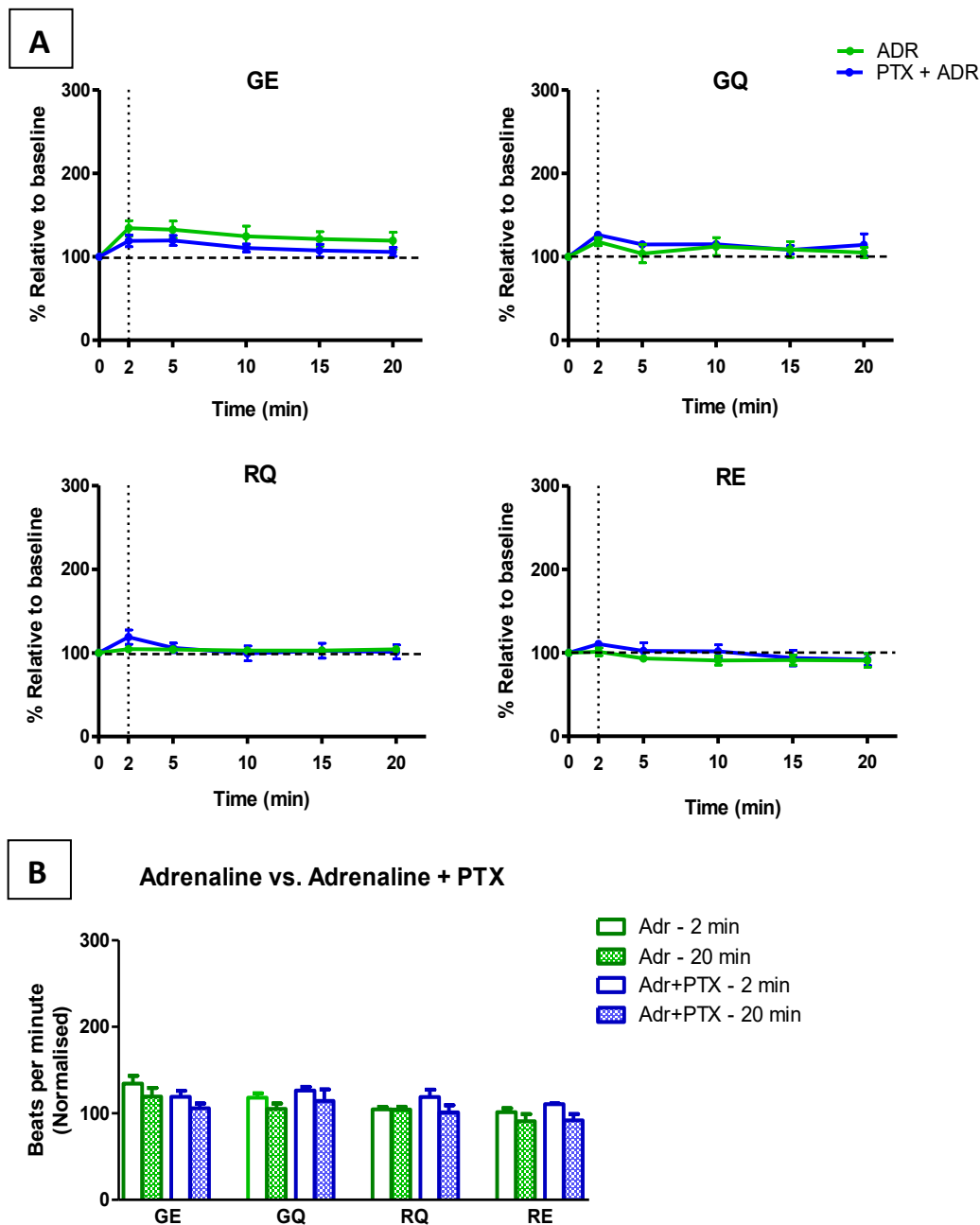


Figure 3. 16 Normalised beating rate in β_2AR haplotypes upon stimulation with adrenaline (green) and adrenaline+PTX (blue)

A) Beating rate of hESC-CMs with GE (6/4), GQ (5/3), RQ (6/4) and RE (7/4) haplotype normalised to basal contractility over 20 minutes of β_2AR stimulation with ISO in the presence of ICI when pre-treated with adrenaline (green), and when hESC-CMs GE (6/4), GQ (5/3), RQ (7/3) and RE (6/3) haplotypes pre-treated with PTX with adrenaline (blue). B) Bar graph showing the beating rate of hESC-CMs with these 4 haplotypes pre-treated with PTX at 2mins and 20mins through ISO stimulation normalised to baseline. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. T = Significant to GE at 2min, # = significant to GE at 20min. (n,N)=cells/batches

3.4 DISCUSSION

The β_2 subtype of the adrenergic receptor is a type of membrane bound GPCR and has an important role in cardiovascular health and disease. The β_2 AR is highly polymorphic where the exonic variants of the N-terminus, Arg16Gly (R/G) and Gln27Glu (E/Q), are functionally relevant; and although β_2 AR is a well-studied receptor, the influence of these polymorphisms on cardiac function is not fully understood. The focus of this chapter was to investigate the influence of the four β_2 AR variants (GE, GQ, RE, RQ) on cAMP signalling and the unique protective property of this subtype through receptor interaction with G_i proteins. This was investigated through assessing changes in contractile activity. Here, the differential responses between the haplotypes have been observed in terms of sensitivity to isoprenaline and receptor desensitisation.

CRISPR-Cas9 gene editing technique was used to develop isogenic hESC-CMs lines of the β_2 AR haplotypes. This technique is a hugely powerful method of editing a target region within the genome; however, it can be time consuming and can have off-target effects. In particular, as the *ADRB2* gene consists of a single exon, it was mandatory for the gene editing to be “foot-print free”. Therefore, a recently developed 2-step editing approach involving CRISPR-Cas9 and *PiggyBac* was used (Kondrashov et al., 2018). This was not only advantageous due to the efficiency of the gene editing protocol as it reduced the number of editing steps, making the protocol was more time efficient, but also is highly specific, ensuring no other modifications within the *ADRB2* exon or its regulatory regions occur. Another benefit of this methods was that 4 isogenic lines of hESCs could be developed, which limited any potential interference from the remainder of the genomic background. As all the lines were created from the same source and have the same genetic background, any differences in responses from the variant in question are a result of the haplotype variants. Furthermore, the developed stem cell lines could be differentiated into the target cell type of interest as a renewable source and could be produced as and when required, although such culture and maintenance can

prove costly. Finally, the use of hESCs provides a cell line of human origin, which although may have some immature phenotypic properties in comparison to adult cells, is beneficial over animal cell lines.

This is the first study to investigate the response of the 4 β_2 AR haplotypes variants (Arg16Gly and Gln27Glu) to isoprenaline by measuring cAMP output. Using FRET microscopy provided real-time/short term responses, and can indicate differences in sensitivity of each variant to receptor stimulation. Firstly, a significantly lower EC_{50} value was recorded for the GE haplotype indicating greater sensitivity to receptor stimulation, closely followed by RE, and the RQ haplotype having the highest EC_{50} value, thus being least sensitive (GE>RE>GQ>RQ). In support of these results, it has been indicated by previous studies that human subjects who were homozygous for the GE haplotype displayed enhanced desensitisation upon continuous infusion of isoprenaline for two hours (Dishy et al., 2001). It may be that because the GE variant presents a greater sensitivity to stimulation that leads to a more prominent decrease in beating rate, thus posing a protective mechanism against prolonged stimulation. The FRET response in this study shows that the E amino acid is present in both the lower EC_{50} variants, so it could suggest that it is responsible for the increased responsiveness whereas the Q amino acid may play a role in reducing responsiveness to isoprenaline stimulation. Which may explain the finding from Dishy et al which shows that individuals who were homozygous for Q27 have less desensitisation and down regulation (Dishy et al., 2001). In addition, the G16 variant may be responsible for lowering the EC_{50} further, regardless of the amino acid at position 27, therefore, this indicates that the G16 variant may be an important player in increasing sensitivity to β_2 AR stimulation and the R27 variant may play a role in reducing sensitivity.

The contractile response of each β_2 AR variant revealed further differences between the haplotypes. IonOptix experiments were performed to record the beating rate of each haplotype variant upon β_2 AR stimulation with isoprenaline. The initial beating rate for all haplotypes except RE was between 50-60bpm; RE had the highest baseline beating rate around 120bpm. Dishy *et al* (2001) report individuals of GQ, RE and GE to have a heart rate of 59, 61 and 67 bpm respectively (Dishy et al., 2001). The basal

beating rate of RE in our study is exceptionally high. Due to the variation in baseline beating rate in this study, the beating rate was normalised to allow a comparison in response to β_2 AR stimulation between the haplotype variants to be made. Upon receptor activation, both the G16 haplotypes showed the greatest increase in beating rate, around a doubling in beating rate. This coincides with the cAMP FRET data as the G16 variant had increased sensitivity to β_2 AR stimulation. This indicates that the amino acid glycine at position 16 may be responsible for increasing sensitivity to stimulation and therefore displaying increased contractile activity. The study by Dishy et al in human subject of these haplotype variants shows more modest increases in beating rate, GQ, RE and GE had a heart rate of 61, 63 and 69 bpm respectively during isoprenaline infusion. This unfortunately highlights the crucial drawbacks of in vitro versus in vivo studies.

Furthermore, in this study, the G16 variants showed significant decrease in beating rate from the 2- to 20-minute period of isoprenaline stimulation with the beating rate returning to basal levels. A slower rate of desensitisation was observed in the RQ haplotype and almost no desensitisation was in the RE haplotype. This is consistent with previous knowledge where G16 shows faster desensitisation and enhanced downregulation (Dishy et al., 2001; Green et al., 1994, 1995).

Inhibitory G-protein (G_i) activity mediates hormonal and neurotransmitter action on the adenylyl cyclase signalling pathway, and so G_i activity is known to influence signal transduction (Brown & Harding, 1992). Previous literature shows that PTX successfully inactivates inhibitory G-proteins (Sunyer et al., 1989). To investigate whether this desensitising response seen in the previous experiment was due to β_2 AR interacting with G_i proteins and therefore switching to a different signalling pathway, the G_i inhibitor PTX was used to treat hESC-CMs prior to β_2 AR stimulation with isoprenaline. The initial increase after β_2 AR stimulation which was seen by 2 minutes of stimulation was still present, but the previous desensitisation of G16 haplotypes was lost. Over the 20 minutes of stimulation, the beating rate did not drop but remained constant, and so no change in beating rate at 2 mins vs 20 mins of stimulation was observed. Although the R16 variants did not achieve as much of

an increase in beating rate as the G16 variants, there was a small yet similar trend seen. This suggests that β_2 AR coupling to G_s does occur upon receptor stimulation, and when cells are treated with PTX, β_2 AR coupling to G_i is inhibited, and so, desensitisation of the response is not achieved. However, the response is very minimal and so an attempt to drive the G_i response further was made.

A previous study has reported that biased agonism is most commonly observed at high catecholamine concentrations, and β_2 AR switching its coupling specificity from G_s proteins to G_i (Heubach et al., 2004) and mediates a β_2 AR- G_i -mediated depression of contraction. An adrenaline-shock protocol was adopted to encourage greater G_i activity in order to understand the possible influence of the haplotype variants on β_2 AR- G_i interaction during cardiac stress. Previously, it has been shown that treatment of hiPSC-CMs with adrenaline increases beating rate and action potential duration (Prajapati et al., 2018), however, any underlying protective mechanism through G_i interaction had not been studied. Here, the adrenaline shock model was applied to hESC-CMs by pre-treatment with adrenaline using a protocol adapted from Paur and colleagues (Paur et al., 2012). Interestingly, when cells were stress-induced prior to β_2 ARs stimulation, the haplotypes containing G16 displayed a significantly reduced beating rate, with the GE variant displaying a significantly lower beating rate after 20 minutes of receptor stimulation. However, the R16 haplotypes showed no significant changes in contraction rate from the basal rate. This again indicates that the G16 haplotypes experience greater desensitising properties under stress conditions. Furthermore, the G_i activity during adrenaline-induced stress surprisingly did not seem to influence contractile activity of any of the haplotypes. This suggests that the G16 haplotype plays an important role in lowering contractile activity during cardiac stress, although G_i may not be the reason for this protection.

Limitations and future work:

In this study, β_2 AR- G_i activity was only identified through contractile properties. In future studies, β_2 AR- G_i signalling could also be assessed through measuring the change in cAMP output using FRET

microscopy after incorporating PTX treatment into the FRET protocols would provide insight into the influence of the switch from G_s to G_i binding on cAMP handling within the cell. p38 MAPK or p13K sensors may also be beneficial to study the G_i activity through other pathways.

In this study, the contractile characteristics of the 4 haplotypes were measured using the IonOptix method. Upon investigating the basal contractile rates of the 4 haplotypes, the RE variant had a beating rate more than twice as fast as the basal rate of other haplotypes. This high baseline rate is not something discussed in previous literature and may be a characteristic of our cell lines in particular. It is unknown why this may be, and requires further investigation in future studies. One major limitation of using the IonOptix method for measuring SC-CMs contractility is that ESC-CMs do not possess a regular myocyte structures, and so, other contractile properties such as cell shortening could not be measured. It may also be beneficial to conduct calcium transient studies within the hESC-CMs β_2 AR haplotype lines which may provide further insight into the effects of the treatments, such as adrenaline shock or PTX.

It may be useful for future studies to investigate how these polymorphisms may determine β_2 AR conformational changes upon agonist and antagonist binding as conducted previously (Ahles & Engelhardt, 2009). Ahles et al investigated the importance of polymorphic sites within the β_1 AR using the β_1 AR itself as a FRET sensor by the placement of YFP and CFP in the third intracellular loop at the C-terminus of the β_1 AR. In this way, the direct activity of the receptor based on its conformational change could be investigated. It would be of great use to extend this method towards understanding the effect of β_2 AR polymorphism on receptor properties.

Furthermore, the drug concentrations used in this study were based on previous work within the laboratory to maintain consistency for relevant comparison. However, maybe a concentration-response curve for adrenaline should have been tested for the stress induction protocol. One previous study reported that a statistically significant change in beating rate was only detected when hiPSC-CMs were treated with adrenaline concentrations greater than 100nM (Calebiri et al., 2013;

Schwenzer et al., 2018). In addition to receptor sensitivity, understanding desensitisation was an important aspect of this chapter. It may have been beneficial to use SNAP-tagged β_2 ARs which would provide visual confirmation of receptor activity upon stimulation and providing insight into receptor downregulation, internalisation and di/oligomerization (Calebiro et al., 2013; Schwenzer et al., 2018).

Summary

In this chapter, we began to explore the properties of the β_2 ARs haplotypes on cAMP handling and β_2 ARs- G_i coupling based on contractile activity. The use of CRISPR/Cas9 proved to be an invaluable method for genetically modifying hESC-CMs to express four of the most commonly found β_2 ARs in the human population. Using these four isogenic cell lines means that any differences in response observed were purely due to the haplotype in question. We see from these results that these four haplotype variants are responsible to a certain degree for defining sensitivity to receptor activation/stimulation, driving differentiation contractile properties. Furthermore, there are signs of differences in β_2 ARs- G_i complexes, influencing desensitisation, between the haplotypes; although there is limited evidence to suggest that this pathway is activated during stress induction in hESC-CMs. Therefore, this chapter accepts the hypothesis that β_2 AR genetic variants do play an important role in regulation of receptor-dependent cAMP signalling and that cardiomyocyte physiology is influenced by the variable G_s/G_i composition. However, additional work is required to investigate other potential mechanisms, such as downregulation, internalisation and di/oligomerization which may be involved. Furthermore, genome wide association studies may prove beneficial for identifying other SNPs which may be in linkage disequilibrium with these variants, thus highlighting other potential SNPs which should be focused on in the future. In the next chapter, we begin to shed light on some of the structural features of hiPSC-CMs, and understand how they may influence their functional properties.

CHAPTER 4: ASSESSMENT OF STRUCTURAL MATURATION OF IPSC-CMs

4.1 INTRODUCTION

In this chapter, development of some of the structural features of iPSC-CMs will be explored. One mechanism described Rusconi et al is through the treatment of cells with a Cav β 2 mimetic peptide (MP) that modulate LTCC trafficking and internalisation, thereby controlling the level of LTCC at the plasma membrane of T-tubules. The phosphorylation status of Cav β 2 can affect protein levels of Cav α 1.2 (Rusconi et al., 2016). How the Cav β 2 MP may be involved in the regulation of a family of genes encoding several other structural and function proteins, particularly of the membrane structures or of sarcomeric assembly, was in question.

CAV2 and CAV3 are essential components of caveolae membrane and may act as scaffolding proteins to organise other molecules involved in cell signalling and maintenance of cell structure. CAV3 is also thought to help regulate calcium levels in muscle cells to control muscle contraction and relaxation (*CAV3 Gene - Genetics Home Reference - NIH*, n.d.). TNNT genes encode Troponin-T (TnT) which is a central player in calcium regulation of the actin thin filament and is essential for cell contraction. Alternative splicing and posttranslational modifications lead to additional structural and functional variations of TnT proteins in order to modulate muscle contraction and relaxation. TnT interacts with other key regulatory proteins of the thin filament (Wei & Jin, 2016). Bridging integrator 1 (BIN1) proteins in the cardiomyocyte membrane functions as a scaffolding protein and has emerged as a critical multifunctional regulator of transverse-tubule (t-tubule) function and calcium signalling in cardiomyocytes. Levels of BIN1 protein will be investigated. Lastly, the adrenoceptor gene expression will also be investigated.

The second method of structural maturation of iPSC-CMs will be through microcontact printing. This method has been described numerous times for controlled geometry for cell growth and have shown that myocytes remodel their myofilament structure in response to the feature geometries (Salick et al., 2014). It has been previously suggested that features with lane widths between 30 μ m and 80 μ m

promoted highly aligned myocytes with significantly increased sarcomeric alignment, cytoskeletal structure, nuclear shape and functionality (Kaji, Takii, et al., 2003; Kijlstra et al., 2017; Lehnert et al., 2004; McBeath et al., 2004; Salick et al., 2014; Thomas et al., 2002).

The shape of adult human myocytes is closely associated with their contractile function. Previously, when NRVMs were plated on patterned substrates to manipulate their length:width ratio, those which had ratios similar to adult myocytes had the best contractile function, and those with ratios of myocytes in failing hearts, had poor contractile performance (Kuo et al., 2012). Furthermore, the myocytes are highly differentiated cells and have unique structural features which are essential in mediating adrenergic signalling transduction (Ravi C. Balijepalli et al., 2006b; Patel et al., 2008). Despite the importance of myocyte structure on cardiac function, the influence of manipulating iPSC-CM structure on caveolae components as well as β_2 AR signalling and its associated PDE activity has not fully been understood.

This chapter aims to investigate the influence of MP treatment on gene expression of structural components of iPSC-CMs. The functional influence on LTCC channels will be observed. The structural influence of micropatterning iPSC-CMs will be assessed along with the functional influence through β AR signalling and PDE activity.

4.2 Methods

4.2.1 Cell culture

hiPSC-CMs were produced and cultured in the Sian Harding lab by Thusharika Kodagoda from hiPSCs (IMR-90) cell line as described in Chapter Two, Section 2.1.1. In this chapter, cells were used at D30-D35 from differentiation (Fig 4.1).

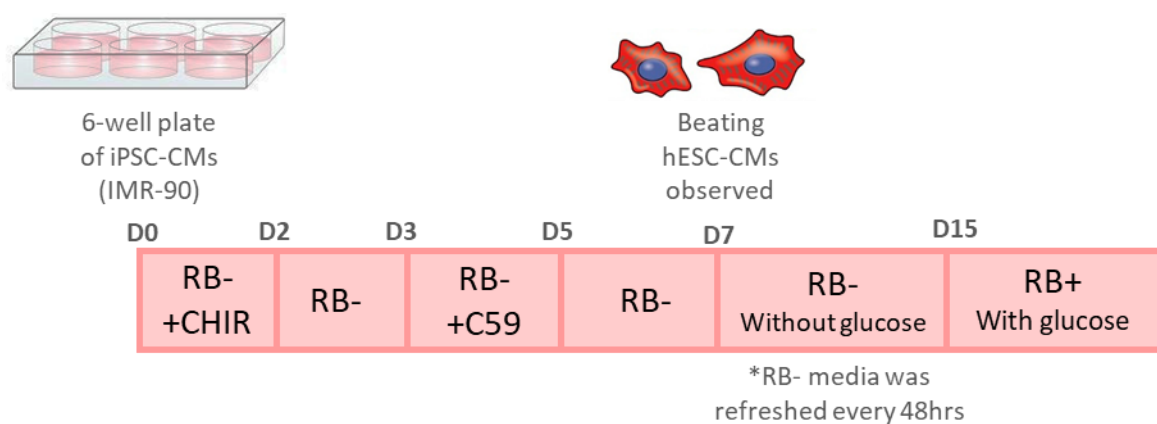


Figure 4. 1 iPSC-CMs (IMR90 cell line) differentiation protocol

4.2.2 Mimetic peptide treatment

The Mimetic peptide (MP) and Scramble (SCR) peptides were a kind gift from Daniele Catalucci from Italy (Rusconi et al., 2016). The protocol of treatment (Fig 4.2) was developed together with Professor Daniele Catalucci. Transfection of iPSC-CMs with SCR or MP was conducted as described in section 2.2.3. The experiment was designed to have a SCR control group for each MP treatment group. Unfortunately, due to the departure of a collaborator, an SCR control was only obtained for the D5 group and the D10, D20 and D30 could not be completed.

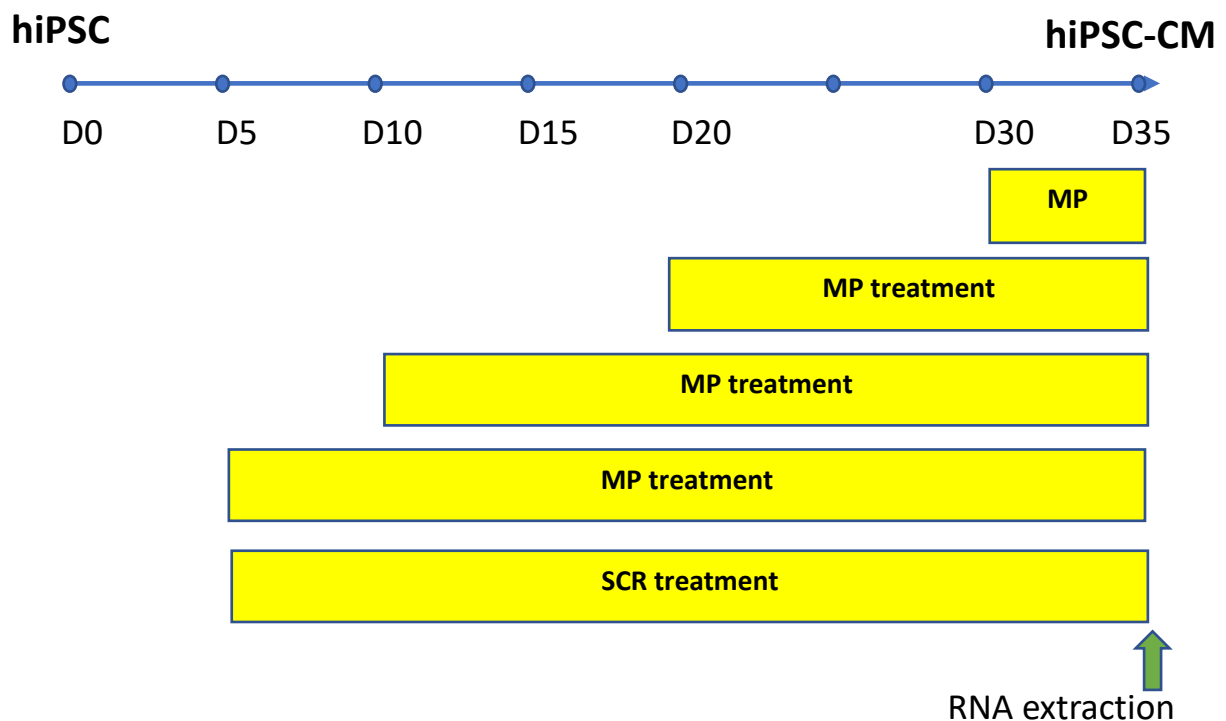


Figure 4. 2 Protocol for MP and SCR drug administration

Protocol depicting the time-points and the duration of MP and SCR treatment of iPSCs throughout the differentiation period into iPSC-CMs. All cells were cultured until D35 at which point they were harvest and prepared for RNA extraction.

4.2.3 Micropatterning of hiPSC-CMs

Poly-di-methyl-siloxane (PDMS) stamps were produced by Ravi Desai and Claire Poulet in A. Oates lab (Desai et al., 2014). They were designed to have 20µm grooves which had a depth of 10µm; the detailed methodology is as described in section 2.2.2.

4.2.4 Immunostaining

D30-D35 iPSC-CMs were seeded on glass-bottom 7mm MatTek dishes at low density for immunostaining. They were cultured for 3-4 days to allow recovery and then prepared for staining as described in Section 2.5.1.

4.2.5 Myofibril alignment analysis

OrientationJ software tool is a series of ImageJ plugins which was used to analyse directionality in the immunofluorescence images. This software characterises the orientation properties of a defined region of interest (ROI) in an image. It computes a histogram indicating the orientation of each structure within the image. For example, an image with an isotropic content will give a flat histogram, whereas an image in which there is a preferred orientation the histogram will display a peak at that orientation.

4.2.6 Transmission Electron Microscopy

4.2.6.1 Reagents and protocol

iPSC-CMs at D30-D35 were prepared for TEM in a confluent monolayer on plastic-bottom, 12-well culture dishes. Culture medium was removed from cell culture dishes; cells were rinsed with PBS then

fixed in 2.5% glutaraldehyde in cacodylate buffer. Further preparation was conducted by Ivan Diakonov as described in Section 2.4.1.

4.2.6.2 TEM Analysis

Analysis of caveolae invaginations was conducted manually. For each treatment, between 11 and 15 cells from 3 to 4 sample preparations were produced. From each cell, an average of 7 frames were taken. The number of caveolae was counted for each frame and then an average for the cell was calculated. This was then used to calculate the average number of caveolae per μm of membrane.

4.2.7 FRET

4.2.7.1 Transfection of iPSC-CMs with pTEV FRET sensor

For the cAMP-specific FRET studies conducted in this chapter, cells were transfected with the FRET sensor plasmid Turquoise-Epac1-Venusvenus (pTEV). The Lipofectamine3000 kit was used. The manufacturer's guidelines were followed with a few modifications to the incubation times as described in section 2.6.5.

4.2.7.2 FRET protocol for assessing $\beta_2\text{AR}$ activity

The activity of $\beta_2\text{AR}$ -dependent cAMP-degrading PDEs in hiPSC-CMs were of interest throughout this chapter. Unpatterned control and micropatterned cells were transfected with pTEV as described above and were all around $\text{D}35 \pm 3$ days at the time of the FRET experiment. After removing cells from the incubator, the culture media was removed and the cells washed gently with room temperature FRET buffer. 500 μL of CGP was added and treated for 10 minutes before FRET recordings were

initiated. The recording was then started and when a stable baseline was obtained, 500μL of 30μM ISO was added to the bath of the dish, then 500μL of IBMX for a final concentration of 100μM. Finally, 500μL of NKH was added to the bath for a final concentration of 10μM. Drugs were only added to the bath once a plateau had been obtained from the previous drug during the experiment. After the final plateau was obtained by NKH, the FRET recording was ended.

CGP	ISO	IBMX	NKH
10 min	10 min	10 min	10 min

Figure 4. 3FRET protocol for assessing β_2 AR activity

4.2.7.3 FRET protocol for assessing phosphodiesterase activity

After β_1 AR inhibition with CGP, β_2 AR was stimulated with ISO 10μM. Then EHNA, 10μM Cilostamide or 10μM Rolipram were added to selectively block PDE2, PDE3 and PDE4. 100μM IBMX was added to the bath for non-selective inhibition of PDEs, followed by 10μM NKH for AC activation to obtain maximal cAMP production in the cell. All drugs were prepared in FRET buffer (Fig 4.4).

10 min	10 min	10 min	10 min	10 min
100nM CGP	30nM ISO	10μM EHNA	100μM IBMX	10μM NKH
		10μM CILO		
		10μM ROLI		

Figure 4. 4 FRET protocol for assessing β_2 AR-dependent PDE activity with the respective drug treatments at the stated final concentrations

4.2.7.4 FRET analysis

Analysis of the FRET results were conducted in Microsoft Excel. The ratio between the fluorescence recorded from the YFP channel and the CFP channel was calculated and plotted to obtain a regular YFP/CFP FRET trace. These results are shown as line graphs with the “FRET ratio (Venus/Turquoise)” plotted. From this the percentage change in the ratio from baseline to the drug treatment in question was calculated. The FRET response from ISO or the PDE inhibitor were normalised to the maximal drop recorded when the cells were treated with NKH. These results are shown as bar graphs plotted as “% FRET change Venus/Turquoise normalised to NKH”.

4.2.8 RT-QPCR

The RNA extraction and QPCR were conducted by Maria Barandalla Sobrados in Daniele Catalucci’s lab at the Humanitas Research Hospital, Italy.

4.2.8.1 RNA extraction

iPSC-CMs were cultured to D30 in a confluent monolayer on glass-bottomed culture dishes. RNA was extracted using the RNeasy Mini kit (Qiagen, CA, USA) from cultured hiPSC-CMs according to the manufacturer’s guidelines as described in section 2.3.1.

4.2.8.2 RT-QPCR PROTOCOL

The RNA extraction and RT-QPCR was kindly run by Maria Barandalla Sobrados at the Human Institute of Research in Professor Danielle Catalucci’s lab in Italy. Gene expression profiles were investigated in iPSC-CMs at D35 of culture. iPSCs were plated and differentiated into cardiomyocytes as outline above and were either untreated or MP/SCR treated as described above. They were then harvested and RNA was extracted as described above. The Qiagen kit was used to perform RT-QPCR as described in section 2.3.2. The experiment was designed to also investigate the expression of Cav1 and TNNT2, however,

due to the departure of a collaborator, this could not be conducted and should be considered for future studies.

Target Gene	Forward sequence	Reverse sequence
Caveolin 1	TCCCTTCTGGTTCTGCAATC	CGAGAAGCAAGTGTACGACG
Caveolin 3	GCGGAAGGAGGTCTAAAGC	AGAGCGAAGGGCCATTGT
TNNT1	GACTACATGGGGGAGGAACA	ACAGCTCCTGGGCTTTCTC
TNNT3	AAGAAGACACCGCAGAGGAG	AGGTCTTTGTTCTGACGCTTCT
Bin1	GCAGGCTCATCTCGTAGCTC	AAACACCTTCTGGGCTTTGA
α_{2a} -Adrenoceptor	ACATCCCCAGTTGTTGGTTT	GGGTGGCCCACTAGGAAG
β_2 -Adrenoceptor	TCCTGGATCACAATGCACAAT	GAGCACAAAGCCCTCAAGAC
β_3 -Adrenoceptor	GACTTTCGAGCGCCTT	CAAGAAGCCCCGTCGAG
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Table 4. 1 Forward and reverse primer sequences used for RT-qPCR

4.2.8.3 Analysis

Prior to using the QPCR data, the melting curves were checked for the presence of a single peak through the cycle which rose above the threshold. This indicated that the amplified products were of a single species and off-targets products or contamination was not occurring. As these did not occur, the data was used for analysis. The data was normalised to the housekeeping gene GAPDH. The fold differences were calculated through using the “ $2^{-\Delta\Delta Ct}$ ” method. Statistical significance was tested through the Students T-test.

4.2.9 Scanning ion conductance microscopy

iPSC-CMs topography images were obtained by scanning the surface of the cells using a borosilicate glass nanopipette with an internal diameter of 100nm, which is mounted onto a three-axis piezo-actuator which can move in the x-y-z direction. It is controlled by a piezo-actuator (Novak et al., 2009). The pipette is mounted into its holder in the vertical direction and hops along the surface of the cell

membrane without touching the cell surface. The vertical and lateral displacements are recorded to build a reconstructed 3D image of the cell surface.

The nanopipette is filled with electrolyte and has an electrode connected to a feedback mechanism which receives information from the ground electrode placed in the bath. SICM relies on the increase of resistance in an electrolyte-containing micropipette when it approaches the non-conductive surface of the cell. The ion current flowing through the pipette is measured and fed back to the piezo-drive that moves the pipette upwards to keep the pipette-surface distance constant. The use of an ion-current feedback mechanism ensures that the distance between the pipette tip and the sample can be maintained, as there is a drop in current when the pipette approaches the cell surface. At the same time the micropipette movement is recorded while the pipette scans the surface of the cell via a serpentine path, and that provides a topographical image of the cell surface (Bhargava et al., 2013; Korchev et al., 1997).

The glass nano pipettes used for scanning mode SICM had a tip diameter of ~100nm. The pipettes were fabricated by mounting on a CO₂ laser-based micropipette puller system with the following parameters: heat (305 and 300), filament (2), velocity (27), delay (180 and 160) and pull (180).

4.2.9.1 SICM analysis

The topographical images obtained from SICM were analysed in two ways. Firstly, the scans were used to calculate the number of membrane invaginations of iPSC-CMs which were counted manually from each image. Secondly, profiles of the invaginations in the surface topography were created to assess the depth and width of the opening of the invagination (Fig 4.5).

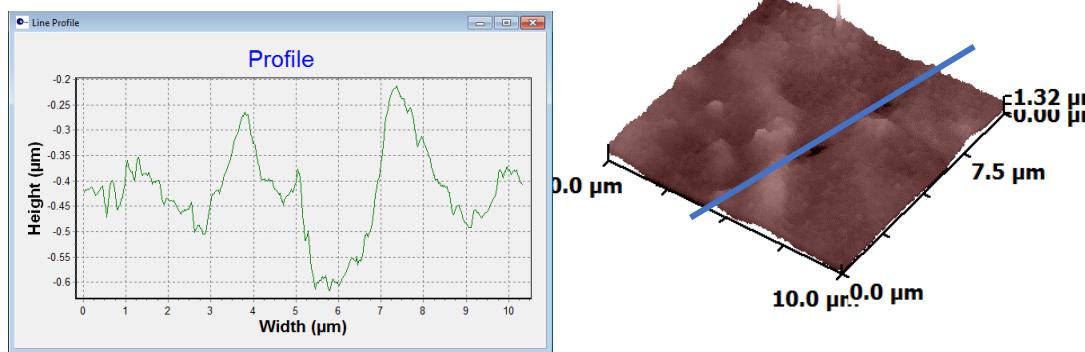


Figure 4. 5 SICM profiles for calculating membrane invagination depth and width

4.2.10 Super-resolution patch-clamp technique

The patch clamp technique was used to study of ionic currents in iPSC-CMs using the “voltage clamp” technique. Once the topographical image was obtained from the SICM, the cell-attached patch-clamp recording technique was used to study L-type calcium channels (LTCC). The computer-controlled piezo was used to progressively clip the tip of the pipette after the topography scan (~100nm internal diameter) to obtain a wider, desired tip diameter for the patch (~300 nm internal diameter) (Bhargava et al., 2013). After clipping the pipette, it could be moved back to a precise coordinate of the known topology. A glass pipette containing electrolyte solutions is tightly sealed onto the cell membrane facilitated by slight suction, indicated by an increase in membrane resistance (referred to as 1GΩ seal), therefore electrically isolating a single patch of the cell membrane. Currents fluxing through the LTCC in the isolated patch region flow through the glass pipette and are recorded by an electrode connected to an amplifier. The resulting current which flows through the electrode is assumed to flow exclusive along the cell membrane. The patch clamp technique allows single L-type calcium channels properties were recorded.

4.2.10.1 L-Type Calcium Channel recordings

L-Type Calcium Channel (LTCC) recordings were conducted using the super-resolution scanning patch clamp with the pipette clipping modification technique. The cell-attached patch clamp recordings were performed at room temperature, with the pipette filled with the internal pipette solution (Table 4.3) and the media of the iPSC-CMs in MatTek dishes was replaced with the external bath solution (Table 4.2).

Compound	mM	FW	g/1000 ml	g/500 ml
K-gluconate	120	234.25	28.11	14.055
KCl	25	74.55	1.86375	0.931875
MgCl ₂	2	203.31	0.40662	0.20331
CaCl ₂	1	147.00	0.147	0.0735
EGTA	2	380.40	0.7608	0.3804
Glucose	10	180.16	1.8016	0.9008
HEPES	10	238.30	2.383	1.1915

Table 4. 2 External solution for single L-type calcium channels recording.

Compound	mM	FW	g/1000 ml	g/500 ml	g/50 ml	g/25 ml
BaCl ₂	90	244.28	21.9852	10.9926	1.09926	0.54963
Sucrose	10	342.3	3.423	1.7115	0.17115	0.085575
HEPES	10	238.30	2.383	1.1915	0.11915	0.059575

Table 4. 3 Internal (pipette) solution for single L-type calcium channels recording.

4.2.10.2 Data analysis

The occurrence is described as the probability to detect channel activity in every patch-clamp recording. This is calculated as a ratio of the number of channel activities observed in each patch. This characterises the distribution of channels long the cell membrane. In comparison to adult myocytes,

which possess T-tubules and crest regions, this separation cannot be made in iPSC-CMs as they do not possess these separate features. Therefore, a single LTCC occurrence value is reported.

4.2.11 Statistical analysis

The statistical tests conducted has been described in each experimental method section. Generally, the statistical analysis used to analyse data obtained for experimental work was Students T-Test, which were performed using GraphPad Prism 5. The statistical significance was set at $p < 0.05$ and data has been reported with $\pm$ SEM (Standard error of the mean).

4.3 RESULTS

4.3.1 CAV2 and CAV3 gene expression through differentiation with MP treatment

RT-QPCR analysis was conducted to investigate whether treatment with MP influenced gene expression of various proteins involved in structural or functional activity of iPSC-CMs as they develop. Initially, CAV2 and CAV3 gene expression was assessed. Overall, both CAV2 and CAV3 showed a gradual increase in the level of gene expression over the 35 days of differentiation in control cells. At D5 of differentiation, there was an undetectable level of CAV2 expression. By D10 CAV2 became detectable and the expression level continued to increase until D35 of differentiation. From D10 to D35, there was almost a four-fold increase in CAV2 expression. The treatment with MP showed a small increase in gene expression in CAV2 at D15, although this returned to basal levels at D25. There were no significant differences detected in comparison to the SCR peptide or the untreated cells (Fig 4.6a).

CAV3 expression also continuously increased over the differentiation period. The expression level at D5 was 14, which rose to 368 by D35. The increase was consistent over the 35-day differentiation period, and seemed to level off between D30 and D35. CAV3 had a much greater increase in the level of expression than CAV2. With respect to CAV3 expression, the treatment with MP in the last 5 days of the differentiation period showed the greatest increase in gene expression with more than a 2-fold increase in expression. When cells were treated with MP at earlier time frames, there was a smaller effect on CAV3 gene expression observed. Similarly, there were no differences in expression levels with SCR treatment at the early stage (Fig 4.6b).

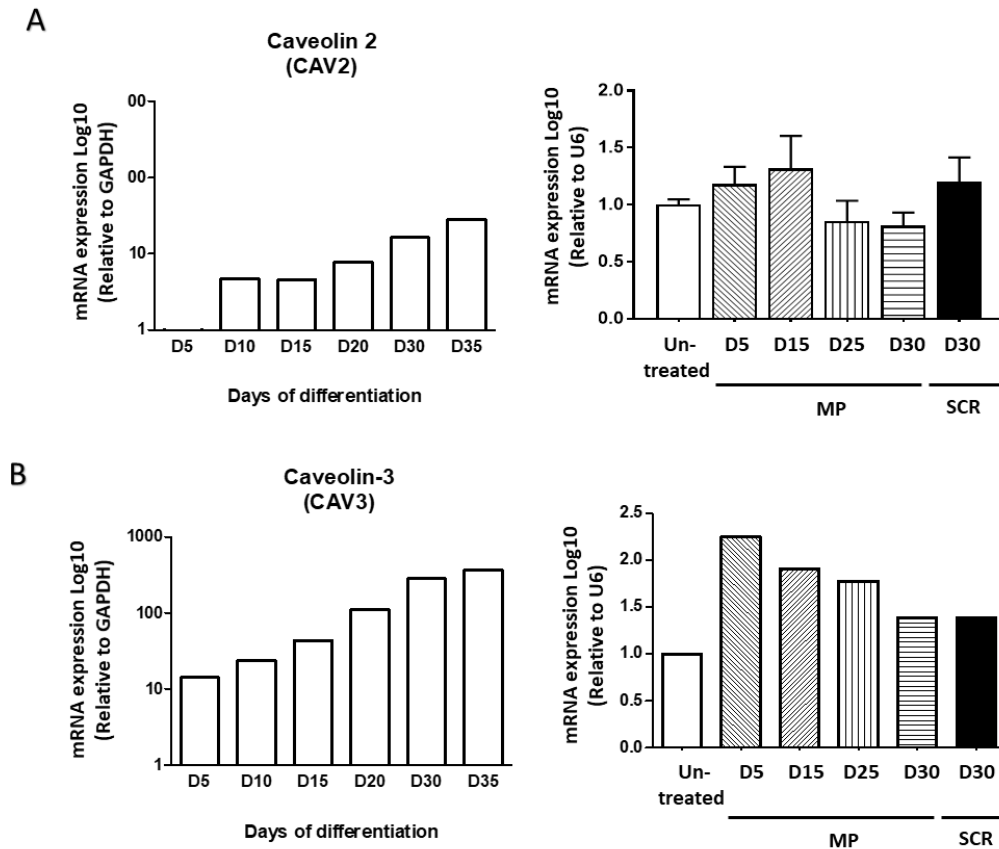


Figure 4. 6QPCR analysis of caveolin2 (CAV2) and caveolin3 (CAV3)

The gene expression of A) CAV2 and B) CAV3 was assessed in iPSC-CMs through the first 35 days of differentiation in control (untreated) cells (left panel) and in those treated with MP or SCR relative to untreated control samples (right panel) (n=1-3).

4.3.2 TNNT1, TNNT3 and Bin1 gene expression through differentiation and MP treatment

The expression level of a few selected structural genes, slow skeletal muscle Troponin T (TNNT1), fast skeletal muscle Troponin T (TNNT3) and BIN1, were also assessed. In control cells, TNNT1 was most greatly expressed at D10 of cell differentiation but decreased throughout the subsequent 10 days of differentiation. At D20 TNNT1 expression was the lowest and remained low at D30. Interestingly, its expression levels increased again by D35. The fluctuation of expression maybe due to the low n-numbers, which may become stabilised as the sample size is increased. When cells were treated with MP, those that received the treatment at D20 of differentiation showed an increase in TNNT1 expression levels, however, the large error bar is somewhat concerning. The other treatment stages did not show any changes in expression, as is seen in SCR and untreated cells (Fig 4.7a).

Interestingly, TNNT3 followed the opposite pattern of expression to TNNT1. Its expression levels dropped dramatically in the first 15 days, although expression levels gradually increased and returned to similar levels at the start of differentiation by D35. When cells were treated with MP, TNNT3 expression was greatest in cells that received the treatment at the end stages of differentiation; though statistical significance was not obtained. Overall, treatment with MP did not alter TNNT3 expression throughout differentiation, and similar expression levels to untreated and control cells are reported (Fig 4.7b).

BIN1 expression was slightly increased at D10 of differentiation with a gradual reduction in expression level over the 35-day differentiation period. Treatment with MP, had a similar effect on Bin1 expression as it did on TNNT1 expression. An increase in expression was detected when cells were treated at D20 of differentiation, although statistical significance was not obtained. In addition, expression levels were lower when cells were treated at earlier stages. Once again, no overall difference was detected in expression levels from untreated or control cells (Fig 4.7c).

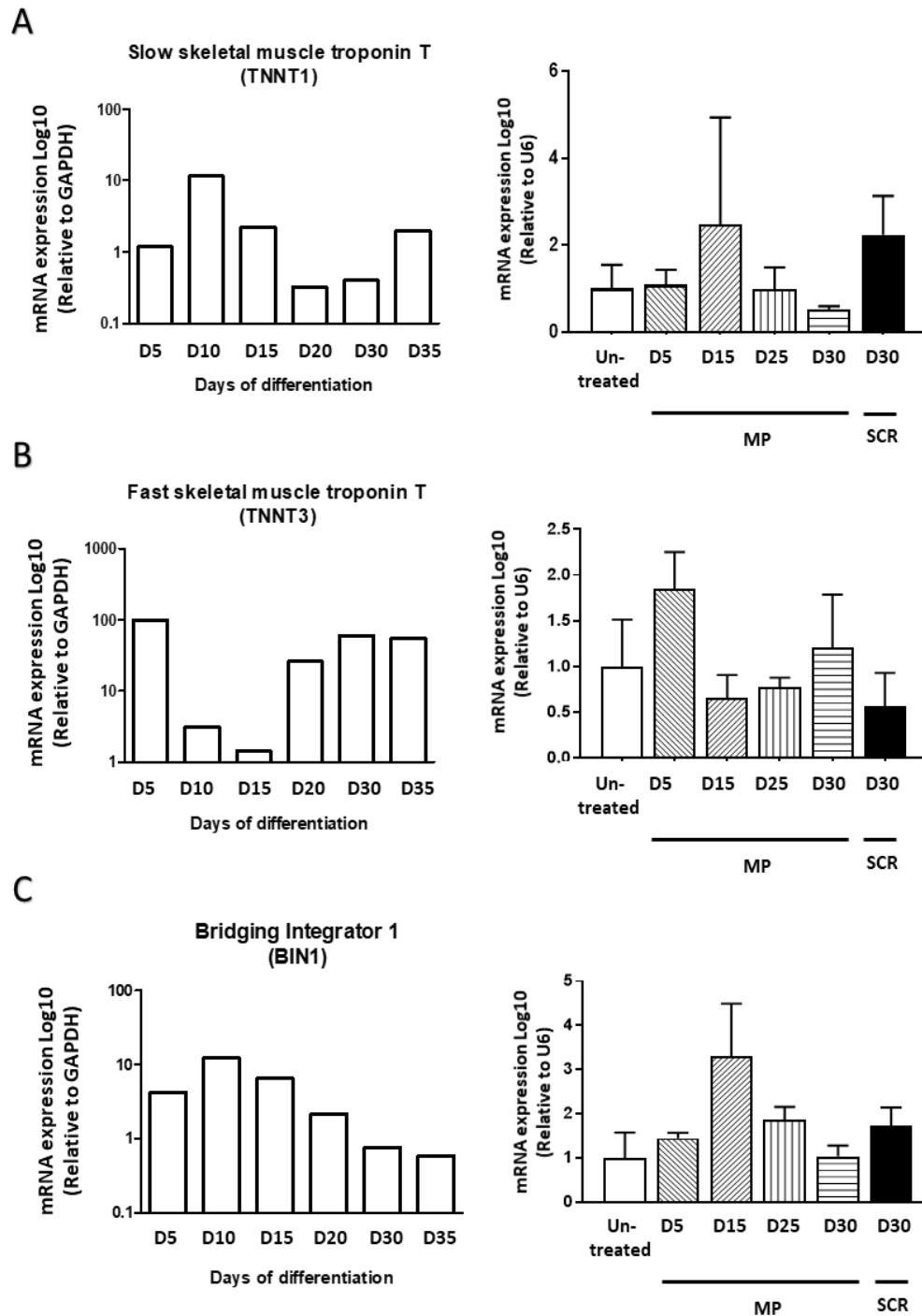


Figure 4. 7QPCR analysis of TNNT1 and TNNT3 and Bin1

The gene expression was assessed in iPSC-CMs through the first 35 days of differentiation in control (untreated) cells (left panel) ($n=1$) and in those treated with MP or SCR relative to untreated control samples (right panel) ($n=3$).

4.3.3 Adrenoceptor gene expression through differentiation and MP treatment

The influence of MP on the expression levels of the adrenoceptors were also investigated. Under control conditions, α_2 AR (ADRA2A) expression levels varied slightly over the 35 days of differentiation. Its highest expression levels were around D10 and D15 of differentiation. Although this decreased by D30, expression level was raised again by D35. However, there was no overall statistically significant change in gene expression. When cells were treated with MP, those treated later in the differentiation procedure, had slightly greater expression levels. SCR treatment also showed an increase in gene expression level. However, no statistically significant differences in expression levels were observed (Fig 4.8a).

Similarly, the relative gene expression of the β_2 AR showed small fluctuations over the differentiation period with no overall statistically significant changes observed. No changes upon MP treatment were observed and expression levels stayed relatively stable throughout MP treatment (Fig 4.8b).

β_3 AR gene expression under control conditions shows a 100-fold increase by day 20 which remains elevated at D30 and D35. β_3 AR expression levels peaked when treated with MP at the late stage of differentiation, showing a 3-fold greater gene expression than untreated cells. However, this is greatly reduced when cells are MP-treated at the other time points. However, n-numbers need to be increased to make firm conclusions (Fig 4.8c).

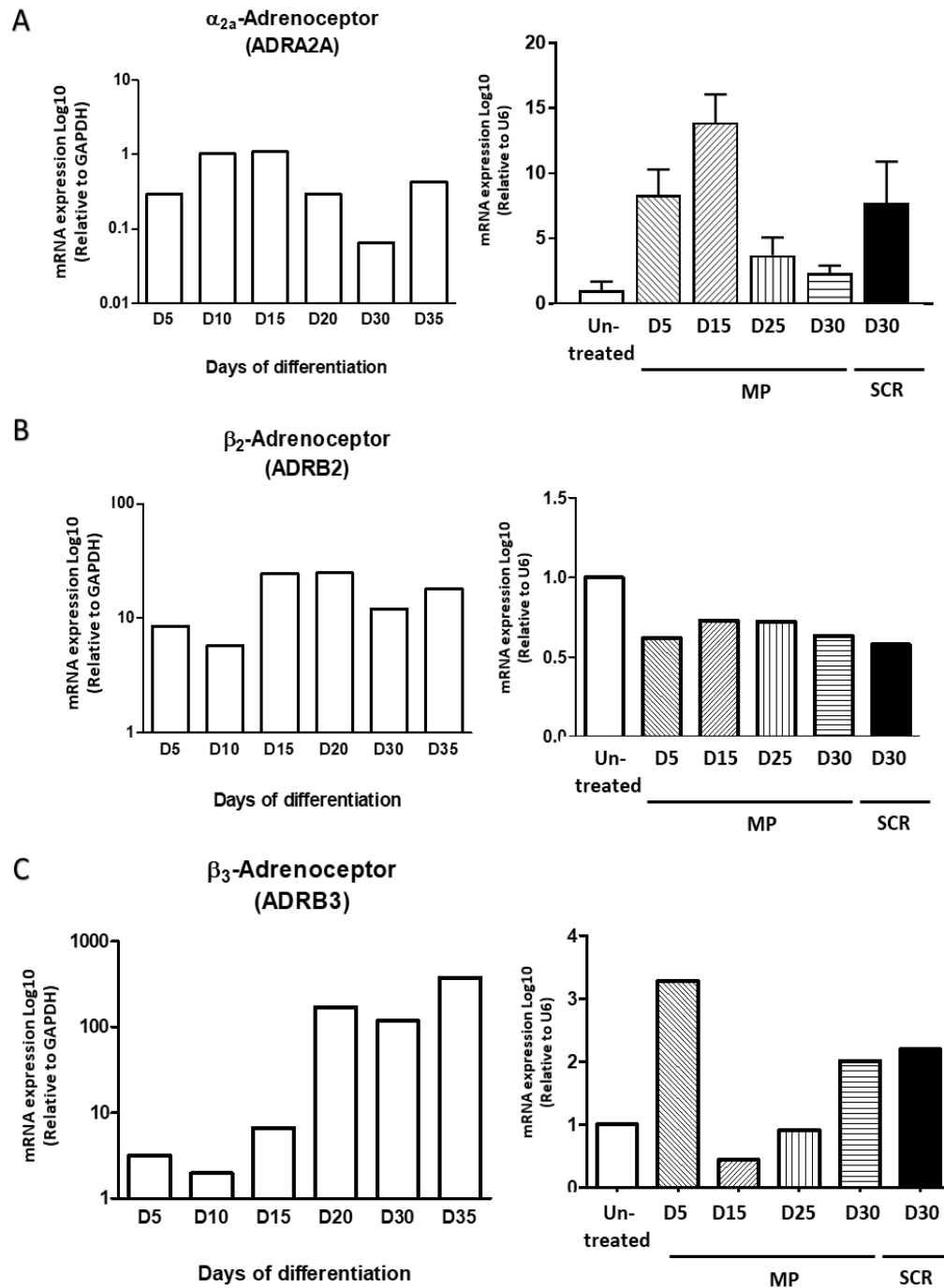


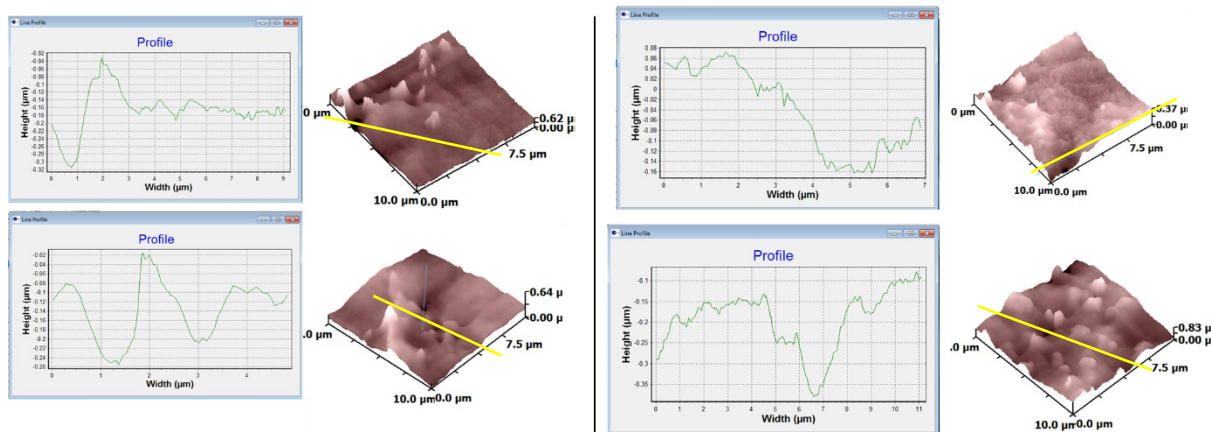
Figure 4. 8QPCR analysis of ADRA2A, ADRB2 and ADRB3

The gene expression was assessed in iPSC-CMs through the first 35 days of differentiation in control (untreated) cells (left panel) ($n=1$) and in those treated with MP and SCR relative to untreated control samples (right panel) ($n=1-3$).

4.3.4 Assessment of iPSC-CMs topography upon peptide treatment

It has been suggested that the administration of MP peptide encourages T-tubular microdomain development. To understand the extent of possible T-tubule “invaginations” in iPSC-CMs, cells were treated with either the MP or SCR peptides. Cells were then prepared for SICM imaging. 10 x 10µm scans of the cell surface were taken from which cell profiles were produced, indicated by the line (Fig 4.9).

SCR treated cells:



MP treated cells:

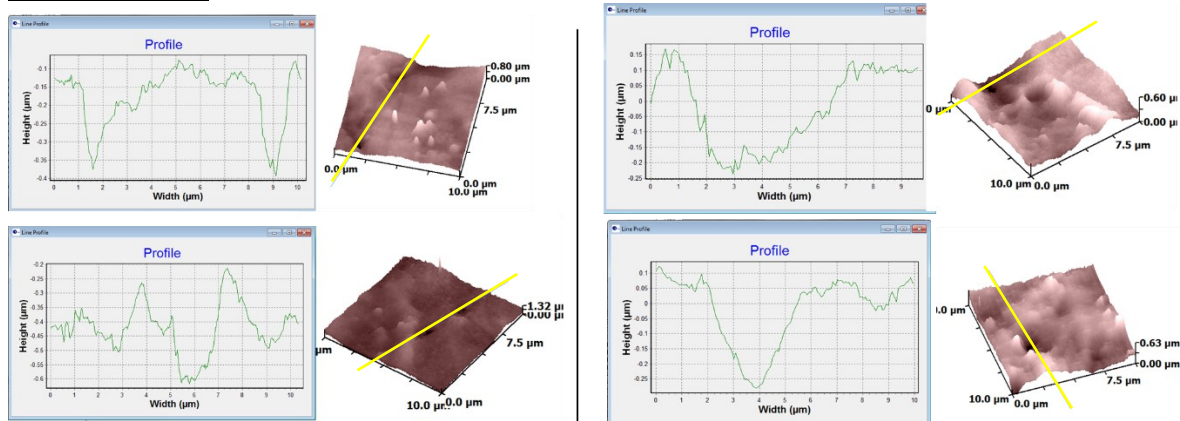


Figure 4. 9Representative SICM images and profile analysis of SCR or MP treated iPSC-CMs

Representative 10µm x 10µm SICM images to depict membrane topography of scramble- (SCR) and mimetic peptide- (MP) treated iPSC-CMs. The blue lines indicate where the respective profiles were produced from. The profiles indicate the depth/invaginations of the membrane along the drawn line.

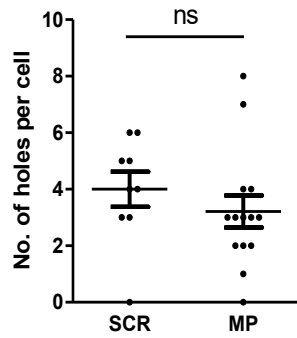
4.3.5 Assessment of MP administration to iPSC-CMs properties

To assess the effect of MP administration for 4 days on the structural maturation of iPSC-CMs, the density and depth of any membrane invaginations was investigated using the SICM images. Firstly, the number of invaginations on the cell surface was calculated. SCR treated cells had an average of 4 ± 0.8 invaginations per cell, and when treated with MP, cells displayed an average of 3.2 ± 0.6 invaginations per cell. On average, 30% of SCR-treated cell scans and 39% of MP-treated cell scans showed the presence of membrane invaginations. This indicates that iPSC-CMs of the IMR cells line more likely than not, do not display membrane invaginations and generally have a relatively flat cell surface. Furthermore, MP treatment is not influencing the number of invaginations of the iPSC-CMs as there was no statistical difference found between MP and the control peptide SCR (Fig 4.10).

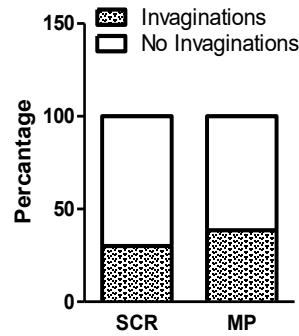
The impact of MP treatment on the cell volume was also assessed. Cells treated with SCR had an average volume of $3.8 \pm 0.8 \mu\text{m}^3$, and those treated with MP had a slightly lower average volume of $2.7 \pm 0.6 \mu\text{m}^3$. No statistically significant difference was observed between these. This indicates that MP treatment does not influence the overall size of the cells, as an increase in membrane fold may correlate with reduced cell volume.

Lastly, the depth of plasma membrane invaginations was assessed. Cells treated with SCR displayed a depth of $2.1 \pm 0.2 \mu\text{m}$ of membrane invaginations, and a depth of $3.2 \pm 0.2 \mu\text{m}$ of membrane invaginations of cells treated with MP. Interestingly, a significant increase in the depth of the invaginations from SCR to MP treated cells was detected ($p < 0.05$). This suggests, that although MP treatment is not influencing the number of invaginations that exist on iPSC-CMs, the peptide treatment is playing a significant role in enhancing the depth of pre-existing invaginations (Fig 4.10).

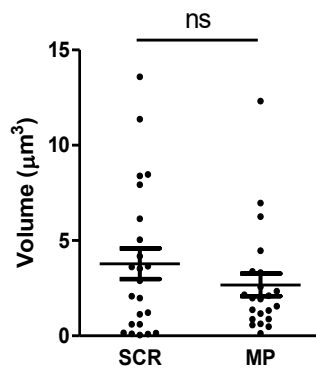
No. of invaginations per treatment



No. of invaginations per treatment



Cell volume



Invagination depth

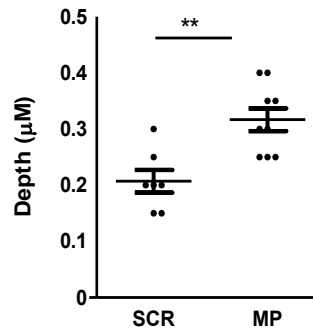


Figure 4. 10 Profile analysis of SCR or MP treated iPSC-CMs

Assessment of the cell volume, no of surface invaginations and depth of invaginations in D30 iPSC-CMs treated with SCR and MP for 4 days. $**p < 0.01$, ns= not significant. Data are presented as mean $\pm$ SEM.

4.3.6 Assessment of LTCC activity in iPSC-CMs

After generating a topographical image of the cell surface by SICM, the pipette tip diameter was increased by using the clipping technique. The pipette was then used for the assessment of single LTCC recordings. The properties of the LTCC was then explored through assessing channel occurrence and density. In adult myocytes, LTCCs were predominantly recorded in T-tubules (30% of successful patches). In the crest region, LTCC activity was recorded at a much lower level (20% of successful patches showed channel activity) (Fig 4.11b). In comparison to adult myocytes, iPSC-CMs showed a much greater occurrence rate (56% of the time, channels were found from successful patches). As described above, previous studies report that iPSC-CMs did not display T-tubule or crest regions; therefore, patches in this study were made on the general membrane region. The density of LTCC was also calculated in the adult myocytes and iPSC-CMs. Channel density in iPSC-CMs was similar to the density observed in the T-tubule region of adult myocytes. LTCC density was much lower in the adult crest region. This indicates that LTCCs cluster in the T-tubules of mature adult myocytes (Fig 4.11c). Due to limited time for this project, the effect of MP treatment on LTCC could not be investigated, and would be an exciting avenue to explore in future studies.

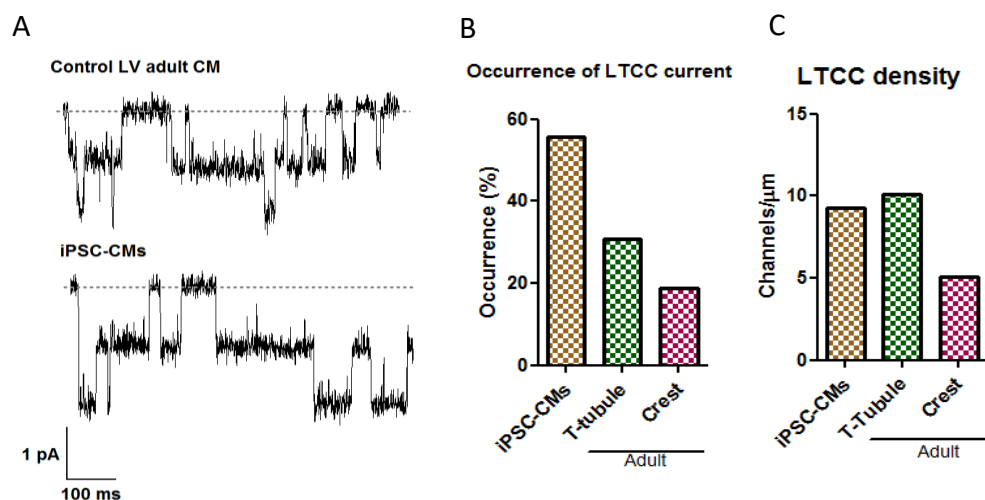


Figure 4. 11LTCC activity and density in iPSC-CMs vs. adult left ventricular myocytes

A) Representative single channel trace showing LTCC activity in left ventricular adult myocytes and iPSC-CMs. B) The chance of obtaining an LTCC current (% occurrence) in iPSC-CMs and human left ventricle T-tubule and crest regions. C) LTCC density in iPSC-CMs and adult myocytes.

4.3.7 Micropatterning iPSC-CMs using PDMS stamps

The second method of structural maturation assessed in this chapter was by microcontact printing. 1cm x 1cm PDMS stamps which had a micropattern width of 20µm were developed and were a gift from as Ravi Desai at the Francis Crick Institute. D30 iPSC-CMs were plated and cultured for 4 days before imaging to allow for enough time for cells to attach to the fibronectin coated lanes. Most unattached cells were washed away with other debris at on the second day after plating when media was refreshed. A further 2 days were given for adhered cells to take their full shape by filling in any gaps, similar to what has been shown before (Koivumäki et al., 2018). Imaging analysis was conducted by taking 2D brightfield images and 3D SICM images of control (unpatterned) and patterned cells.

Unpatterned cells were seen to grow/spread in all orientations with no specific alignment and based on seeding density, sometimes forming aggregates or compact monolayers. Individual cells and monolayers experienced spontaneous contractions. They consisted of cells of all shapes, sizes and orientations. Patterned cells were aligned along the 20µm pattern lane (Fig 4.12a).

The patterned cells grew within the restricted region with more uniformity, elongated alignment and directionality. When cells were plated at higher densities and cell plating was slightly more compact, they occasionally bridged across to adjacent lanes and some overlapping was observed (Fig 4.12a). Contractions were also observed in patterned cells in single cells which were more synchronous when cells were in contact with adjacent cells. Similarly, SICM revealed that unpatterned cells had disorganised/random cell shape and structure, unlike patterned cells. Furthermore, unpatterned cells tended to be flatter than patterned cells (Fig 4.12b).

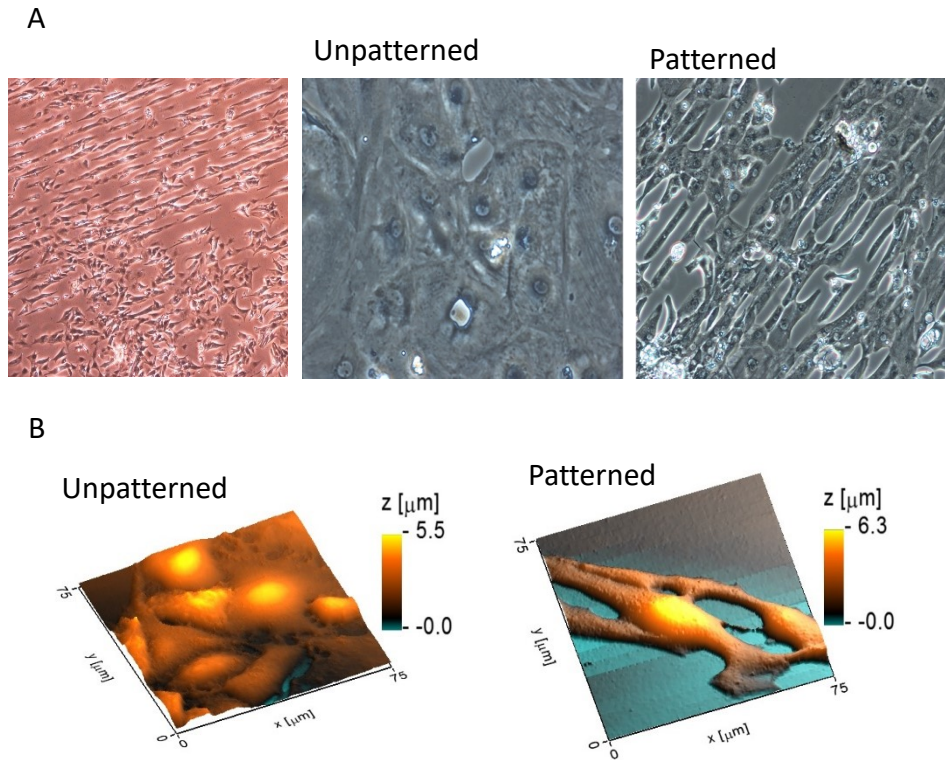


Figure 4. 12 Representative brightfield and SICM images of unpatterned and patterned iPSC-CMs.

Unpatterned cells have no specific alignment or structure, whereas patterned cells follow closely to the printed geometries and a more regular aspect ratio. A) Phase images of unpatterned and patterned iPSC-CMs on fibronectin-coated glass bottom dishes. B) SICM images of unpatterned and patterned cells.

4.3.8 Assessment of sarcomeric organisation in unpatterned and patterned cells

The phase and SICM images show improved cellular geometry of patterned iPSC-CMs. The influence of patterning iPSC-CMs was then assessed through studying the structural components of the sarcomere by immunostaining and confocal imaging of the α -actinin and cardiac troponin-T proteins. Patterned cardiomyocytes displayed improved sarcomeric alignment and regularity through greater organisation of the α -actinin and cardiac troponin-T proteins compared to unpatterned cells. Sarcomeric structures within individual cells are seen to align with each other. There is also an indication that the myofilament orientation exists in parallel striations after patterning (Fig 4.13).

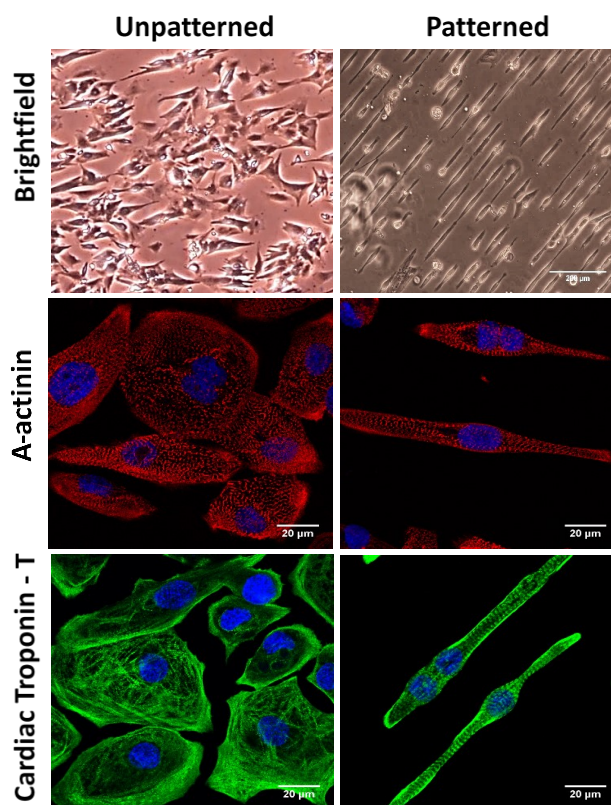


Figure 4. 13 Assessment of sarcomeric development of patterned cells

Micropatterning of iPSC-CMs encourages structural development and myofilament alignment. Brightfield and confocal images of unpatterned and patterned cells stained with α -actinin (red) and cardiac troponin T (green).

4.3.9 Assessment of myofibril alignment in unpatterned and patterned cells

Unpatterned and patterned cells which were stained with α -actinin were then analysed further to assess the degree of myofibril alignment using OrientationJ. As described above, a colour wheel where each angle is represented by a colour is used to colour the protein stained in the cell to indicate the orientation of the myofibril. This is used to produce a histogram of directionality. This process was conducted on unpatterned and patterned cells previously stained with α -actinin.

The pseudo-coloured images, based on orientation of the myofibril, show a greater range and mixed-colouring pattern of the unpatterned cells, with no specific colours being dominant over another. However, the patterned cells clearly show specific regions of organised α -actinin orientation (Fig 4.14b). This was further assessed through the development of a histograms to display the degrees of orientations which could be depicted. It shows that a greater range of colours and a wider distribution of the histogram with multiple peaks for unpatterned cells. On the other hand, patterned cells display a single peak, indicating greater alignment of α -actinin protein in patterned cells (Fig 4.14 c, d).

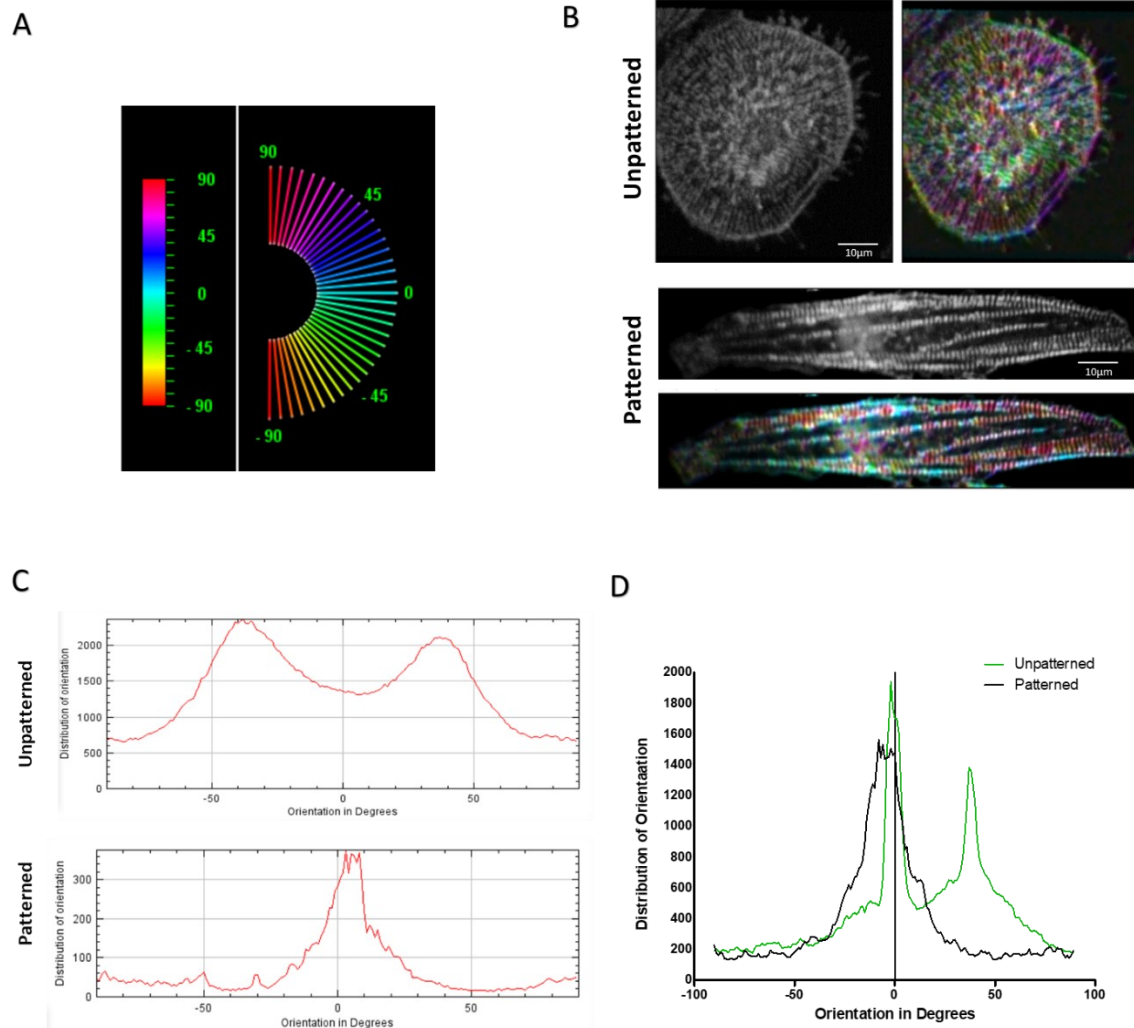


Figure 4. 14 Assessment of myofibril directionality of unpatterned and patterned cells.

Unpatterned cells display a large range of orientations and patterned cells show a single, most frequent orientation of myofibrils, indicating a more uniform alignment. A) Circular colour map coding where each angle is represented by a single colour. B) Grey scale and coloured image panels as a visual representation of myofibril alignment. C) A histogram showing the distribution of orientations in the (B) images. D) Assessment of the orientation of the myofibrils (α -actinin) showing increased alignment in patterned cells represented by a single peak.

4.3.10 Influence of patterning iPSC-CMs on membrane caveolae structures

To further understand the effect of patterning iPSC-CMs on structural development, caveolae components were also assessed as they are crucial components of myocyte structure. Unpatterned and patterned cells in the presence or absence of M β CD were stained for CAV3 and imaged with confocal microscopy. Images were used for calculating protein density.

Analysis revealed that CAV3 protein density was $29.26 \pm 2.3 \text{ cm}^2$ in unpatterned cells and $32.66 \pm 4.2 \text{ cm}^2$ in patterned cells, between no significant difference was observed. This shows that patterning iPSC-CMs has no effect on the density of the CAV3 protein of the cell. However, changes in membrane-bound or cytosolic CAV3 was not assessed which could be important in understanding protein localisation in future studies. Furthermore, treatment with M β CD led to the CAV3 density in unpatterned cells to be $22.2 \pm 2.2 \text{ cm}^2$ and $33.72 \pm 4.2 \text{ cm}^2$ in unpatterned and patterned cells respectively; again, no significant difference was observed in comparison to control/untreated cells.

Although there was no significant difference observed in CAV3 density in unpatterned and patterned cells, interestingly, there were differences observed in the caveolae components present on the iPSC-CMs membrane themselves. Under control conditions, unpatterned cells had around 0.2 ± 0.04 caveolae/ μm and patterned cells had 0.27 ± 0.04 caveolae/ μm . Although there was a small increase, patterned iPSC-CMs showed no significant difference in caveolae number from unpatterned cells. After the treatment with M β CD, caveolae complexes were significantly reduced to 0.11 ± 0.02 caveolae/ μm and 0.09 ± 0.03 caveolae/ μm in unpatterned and patterned cells respectively ($p < 0.05$). This indicates that the M β CD is in fact having an effect on iPSC-CMs membrane caveolae density, without affecting the CAV3 protein itself (Fig 4.15).

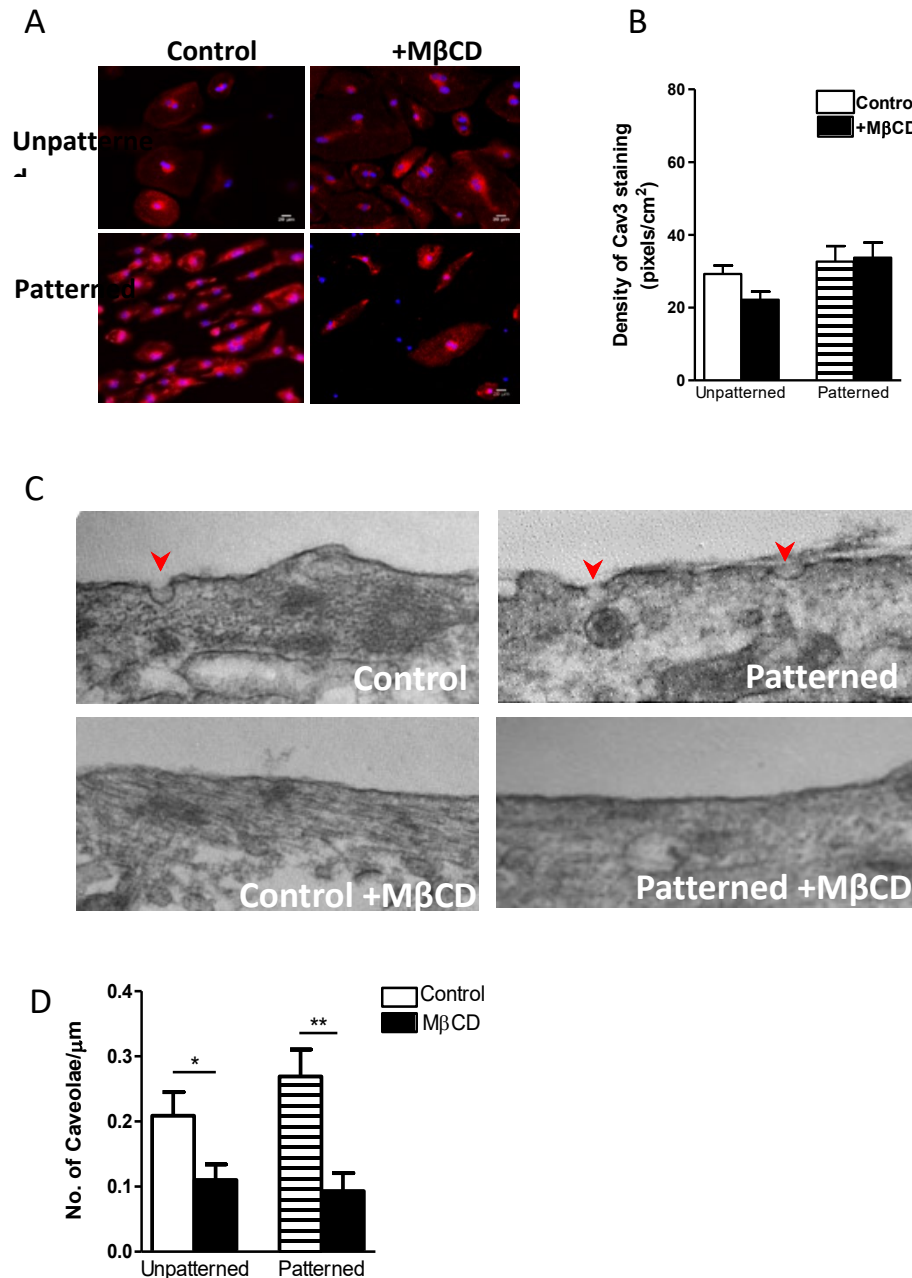


Figure 4. 15 Assessment of patterning on CAV3 protein and caveolae components

The number of caveolar compartments decrease significantly when iPSC-CMs are treated with MβCD. Patterning of iPSC-CMs does not influence CAV3 protein density or the number of surface caveolae. A) Immunofluorescence staining for Caveolin-3 (red) in unpatterned and patterned iPSC-CMs. B) Quantification of CAV3 protein density in unpatterned control (n=18/3) and MβCD treated (n=18/3), patterned control (n=13/3) and MβCD treated (n=6/3). C) TEM images showing surface caveolae (indicated with arrows) in unpatterned and patterned iPSC-CMs. D) Quantification of caveolae per μm of cell membrane in unpatterned control (n=45/3) and MβCD-treated (n=37/3), patterned control (n=15/2) and MβCD-treated (n=9/2) cells. *p<0.05, **p<0.01. Data are presented as mean ± SEM. (n,N)=cells/batches

4.3.11 Analysis of cAMP activity after patterning of iPSC-CMs

Patterning iPSC-CMs has shown to improve structural development through myofibril alignment, though no significant changes were observed in CAV3 protein density or caveolae components. To identify any underlying functional changes of patterning iPSC-CMs with respect to β AR signalling, cAMP activity was measured using FRET microscopy. β_2 AR activity was assessed by stimulation of the receptor with 30nM ISO in the presence of the β_1 AR blocker CGP, with subsequent PDE inhibition with IBMX, then AC activation with NKH.

Unpatterned cells displayed a release of around $45 \pm 4.2\%$ of total cAMP output upon ISO stimulation and $88 \pm 6.2\%$ after PDE inhibition with IBMX. In comparison, patterned cells had a $64 \pm 7.8\%$ cAMP release of total cAMP output and $92 \pm 6.1\%$ after PDE inhibition. Although β_2 AR-cAMP output was higher after receptor stimulation in patterned cells, statistical significance was not obtained, indicating that patterning iPSC-CMs does not influence β_2 AR activity. IBMX significantly increased cAMP output in both unpatterned and patterned cells, suggesting there are functional PDEs in iPSC-CMs actively degrading cAMP ($p < 0.05$) (Fig 4.16).

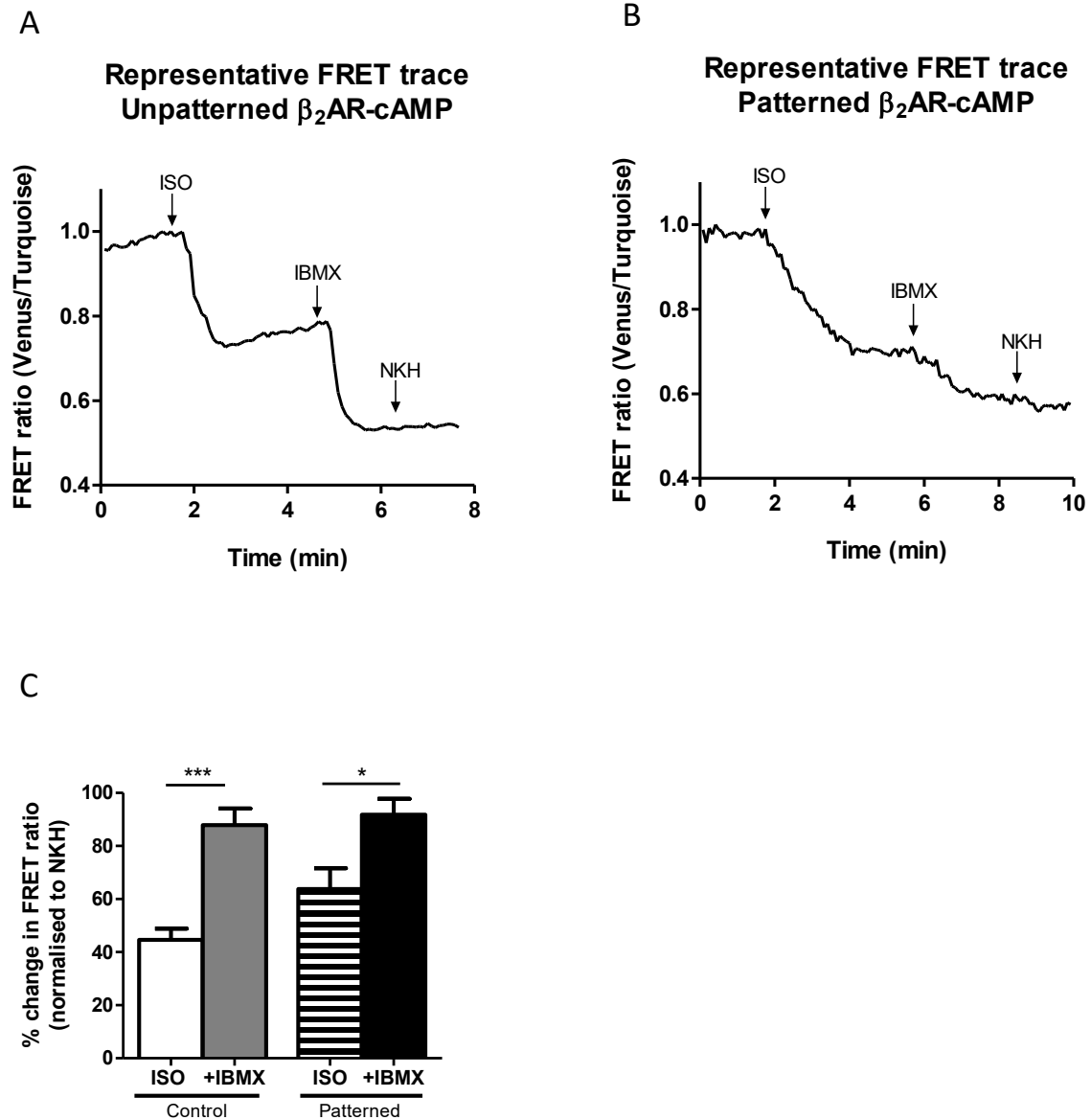


Figure 4. 16 β_2 AR dependent cAMP activity in micropatterned iPSC-CMs

The effect of micropatterning iPSC-CMs on β_2 AR-cAMP activity in control ($n=36/6$) and patterned ($8/3$) cells. Cells were stimulated with 30nM isoprenaline in the presence of CGP, with subsequent inhibition of PDEs with IBMX and then AC activation with NKH. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace of unpatterned cell. B) Representative FRET trace of patterned cell. C) % change in FRET ratio normalised to maximal cAMP output by NKH treatments. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, *** $p<0.001$. n =cells/batches

4.3.12 The effect of patterning on β_2 AR-cAMP dependent PDE2, 3 and 4 activity

As previous results showed significant increases in cAMP output after IBMX treatment, indicating underlying PDE activity, the activity of the major cAMP-degrading PDEs (PDE2, 3 and 4) was assessed using the selective inhibitors EHNA, Cilostamide and Rolipram respectively after ISO stimulation. In control, unpatterned conditions variable responses on PDE2, 3 and 4 inhibition were obtained. There was a $50.96 \pm 5.1\%$, $30.43 \pm 5.2\%$ and $69.33 \pm 4.2\%$ cAMP output recorded upon EHNA, Cilostamide and Rolipram inhibition respectively. PDE3 seemed to have the lowest influence on cAMP degradation, whereas PDE4 shows to have the greatest activity. However, possibly due to low n-numbers, statistical significance was not achieved. In patterned cells, cAMP output increased to $62.36 \pm 5.6\%$, $71.08 \pm 7.6\%$ and $90.76 \pm 4.7\%$ respectively after the addition of EHNA, Cilostamide, and Rolipram. Though this was not a significant increase for PDE2 and PDE4 activity, there was a 2-fold increase in PDE3-dependent cAMP output produced by patterning ($P < 0.05$) (Fig 4.17).

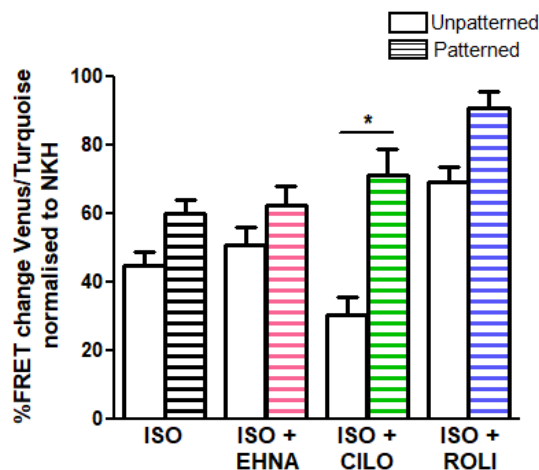


Figure 4. 17 β_2 AR dependent PDE2, 3 and 4 activity in micropatterned iPSC-CMs

The effect of micropatterning iPSC-CMs on β_2 AR-cAMP and its associated PDE activity. Cells were stimulated with isoprenaline in the presence of CGP, with subsequent inhibition of PDE2, PDE3 or PDE4 with EHNA, cilostamide or rolipram. Unpatterned: ISO ($n = 36$), EHNA ($n = 7$), CILO ($n = 20$), ROLI ($n = 28$). Patterned: ISO ($n = 20$), EHNA ($n = 5$), CILO ($n = 7$), ROLI ($n = 8$). The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$

4.4 DISCUSSION

This chapter explored two methods of iPSC-CMs maturation which have previously been indicated to improve cell morphology and structural features.

Firstly, the use of MP throughout iPSC-CMs differentiation was investigated to identify any involvement in the regulation of a family of genes encoding several other structural and function proteins. There was some variability detected in the expression levels of the genes tested, however, due to low n-numbers firm conclusions cannot be made. At this initial stage, it seems that MP treatment may not directly be involved in regulating the expression levels of these genes, however, this still remains to be investigated in depth. Furthermore, we observed that treatment with MP interestingly enhances the depth of any existing invaginations along the iPSC-CMs membrane. However, depicting the exact depth of the invaginations by SICM is limited by the diameter of the pipette as it will not be able to resolve invaginations narrower than its tip. Therefore, to further clarify the depth enhancement, membrane staining and imaging should be utilised. There is previous evidence of invaginations of the sarcolemma of the surface membrane of early-stage iPSC-CMs (Maxwell & Xu, 2018; Ronaldson-Bouchard et al., 2018b). They suggest that with intensity-based training (i.e. constant electrical simulation for several weeks in culture) can enhance the development of these surface invaginations. This was depicted through the use of wheat germ agglutinin (WGA) staining. Further clarification on how MP effects the depth of these membrane invaginations should also be investigated using such staining methods.

Investigation of changes in structural protein expression during iPSC-CM differentiation were also assessed. We find that caveolin2, caveolin3, ADRB1 and ADRB2 increase throughout the 35 days of differentiation. This is in accordance with previous literature (Jung et al., 2016; Haodi Wu et al., 2015). RNAseq data also shed light on the expression levels of various structural proteins as well as numerous ion channels (Karakikes et al., 2015a; Z. Zhao et al., 2018). Zhao et al show that ADRB1 RNA is present

in iPSC-CMs at lower levels than ADRB2. Upon investigation of TNNT1 and TNNT3, changes in expression levels were not conclusive. Future studies should also consider investigating TNNT2 gene expression levels as the protein encoded by this gene is located on the thin filament of striated muscles and regulates muscle contraction in response to changes in intracellular calcium ion concentration. Furthermore, in this thesis we find that BIN1 is expressed at its greatest at D10 of differentiation, however, this tends to decrease over the 35-day differentiation period. It has been shown in previous literature that BIN1 overexpression during hESC-CM differentiation promotes T-tubule development from D10 to D30, and encourages Cav1.2 channel clustering and coupling with BIN1 (de La Mata et al., 2019). Expression levels of Cav1.2 proteins in iPSC-CMs has also been highlighted to be within a similar range to human ventricular myocytes (Lam et al., 2019).

This study preliminarily explores LTCC functionality in iPSC-CMs. We find that iPSC-CMs have similar density of channels in comparison to adult myocytes. Previously, it has been depicted that LTCC density decreases in failing hearts (Shaw & Colecraft, 2013). One other study also reports that iPSC-CMs from monolayer cultures and those of EHTs displayed similar L-type calcium currents and densities to human adult myocytes (Uzun et al., 2016). This supports the findings in the present study.

The environment in which all the functional components of mature adult myocytes operate, such as excitation-contraction coupling, membrane voltage regulation, signalling cascades, maintenance of ion currents of the membrane and action potential waveforms, differs substantially from that of naïve or immature myocytes. hESC-CMs and iPSC-CMs are reported to be much smaller than adult myocytes and generally have a round or polygonal shape, and also lack a regular ultrastructure and T-tubule network (Karakikes et al., 2015b). This prevents development of mature functional characteristics and so, iPSC-CMs are recorded to have spontaneous beating, depolarised diastolic membrane potential, flat action potential duration and slow Ca^{2+} signalling (Kaji, Takii, et al., 2003; Kijlstra et al., 2017; Lehnert et al., 2004; McBeath et al., 2004; Salick et al., 2014; Thomas et al., 2002).

In this study, the second method of structural maturation which was explored was microcontact printing to pattern iPSC-CMs with the aim to physically manipulate their structural features and to investigate the effect on their functional properties of the β_2 AR signalling system. iPSC-CMs were patterned on lanes of 20 μ m in width. Although it had been suggested that lane widths between 30 μ m and 80 μ m promoted structural and functional characteristics (Atmanli & Domian, 2013; Kijlstra et al., 2017; Kuo et al., 2012b; Leu et al., 2001; Snir et al., 2003b), several studies have reported that smaller lane sizes are more effective for single cell alignment of less developed myocytes, such as those of neonatal or SC-CMs (Bedada et al., 2016; Kuo et al., 2012b; Salick et al., 2014). Therefore, due to the small size of iPSC-CMs, narrower lanes of 20 μ m were selected.

Upon assessment of structural maturation of iPSC-CMs based on sarcomeric alignment and organisation, this study shows significant improvements of patterned cells. α -actinin staining displayed clearer and more parallel sarcomeric features than non-patterned cells. These results are in accordance with previously published literature, showing that iPSC-CMs and rodent myocytes cultured in similar ways display improved α -actinin proteins alignment along the Z-line, thus had improved sarcomeric organisation (Koga et al., 2003).

On many occasions, the improvements to the internal structure of iPSC-CMs have been reported, the influence of membrane structure has not been explored, and so investigation into membrane caveolae compartments was conducted. Previous studies show that CAV3 protein is an important scaffolding protein and is located throughout the sarcolemma, and particularly within caveolae plasma membranes (Cohen et al., 2004). In this study, upon staining patterned iPSC-CMs for CAV3, no differences in protein density or membrane distribution were detected. Furthermore, TEM analysis also did not reveal any significant alterations in caveolae components after patterning iPSC-CMs. Using M β CD to deplete membrane caveolae, reduced caveolae number significantly as expected. This shows that CAV3 protein is forming functional complexes with cholesterol in iPSC-MCs and can successfully form caveolae compartments, and sequestering membrane cholesterol destroy caveolae

compartments. However, the uniform alignment of CAV3 observed in adult myocytes is not observed from this study. This indicates, that despite the positive influence patterning iPSC-CMs has on internal structural maturation, not all structural properties can be enhanced in this way.

The influence of patterning iPSC-CMs on β AR activity was then assessed. Since no changes in caveolae properties were observed, one would expect that major differences in signalling properties would not exist, as was the case. A trend of increased β_2 AR-cAMP output was observed, although statistical significance was not obtained. There was a significant influence of PDE activity observed in iPSC-CMs. Upon assessment of downstream PDE activity, PDE2 and PDE4 were responsible for regulating 50-70% of cytosolic cAMP activity. There were no differences in PDE2 and PDE4 activity detected in patterned cells. Interestingly, PDE3 was responsible for around 30% of all cAMP activity. This was significantly increased in cells which were patterned. The reason for this change in PDE3 activity is not fully elucidated in this study and needs to be further explored.

Limitation and future work

Although treatment with MP offers some interesting preliminary insights, a low n-number prevents firm conclusions from being made. It would be important to repeat these experiments to ensure the conclusions hold correct. In particular, gene expression data tends to be very variable, thus having a larger data set is essential.

Despite there being a greater T-tubule depth in MP treated iPSC-CMs, the exact increase in depth cannot be fully characterised by SICM alone. This is because the depth analysis from SICM profiles is limited to the diameter of the pipette tip, therefore, potentially deeper invaginations would not be detected. To overcome this, membrane staining and confocal microscopy (or TEM) should be utilised.

This would provide a more detailed understanding of how far the membrane invaginations penetrate into the cell.

Patch clamp studies had briefly highlighted LTCC properties in iPSC-CMs. Future work should investigate whether these properties remain similar within the membrane invaginations, and whether MP promotes their clustering as is described in adult myocytes. Furthermore, as BIN1 and JPH2 proteins are essential proteins of T-tubules, overexpression of such proteins may facilitate invagination development and LTCC localisation. This would be an interesting aspect to explore in iPSC-CMs.

Patterning iPSC-CMs showed some promising improvements in structural development. However, patterned cells were only present at low densities, i.e. in single-cell populations with gaps within them. Functional properties of myocytes may vary dramatically from single cell cultures to those grown in a monolayer. Future patterning protocols should be developed to potentially allow patterned cells to be grown in monolayer cultures, as functional output can differ upon interactions with neighbouring cells.

iPSC-CMs grown on patterned cells showed greatly elongated cell structure and enhanced contractile apparatus. One important feature of patterned iPSC-CMs to be studied is their ability to contract along the long axis, similar to adult myocytes. This would improve our understanding of myocyte shortening. Furthermore, along with contraction studies, calcium handling properties should also be considered as they are a major contributor of myocyte function and indicator of myocyte health.

Summary

In summary, this chapter highlights how the expression of a few crucial structural proteins of myocytes vary throughout iPSC cardiac differentiation. Two methods of proposed maturation techniques were explored. However, despite some positive influences observed, these methods may still require further optimisation as maturation still remains incomplete in some factors. This chapter accepts the hypothesis that iPSC-CMs possess limited and underdeveloped structural properties and hence display an immature β_2 AR-derived cAMP signalling. However, it rejects the hypothesis that the encouragement of controlled, structured growth through micropatterning will positively effect β_2 AR-derived cAMP signalling. The progression of this study will be into the functional caveolar microdomains of iPSC-CMs as these are another crucial component which play a role in determining β_2 AR signalling functionality.

CHAPTER 5: ROLE OF CAVEOLAE ON β_2 AR-cAMP COMPARTMENTATION IN iPSC-CMs

5.1 INTRODUCTION

In the previous chapter, attempts of iPSC-CMs structural maturation through physical manipulation by $\text{Ca}_v\beta 2$ mimetic peptide treatment showed little change on the expression level of various crucial structural proteins which have previously been identified as hallmarks of iPSC-CMs maturation. Despite this, there was an indication of enhanced membrane invagination depth, which needs to be further explored. Upon physically manipulating the iPSC-CMs by applying a method of patterning to the cells, structural maturation through enhanced cellular alignment and intracellular organisation of structural proteins was observed. However, little changes were observed upon investigating $\beta_2\text{AR}$ -dependent cAMP signalling and PDE2, 3, and 4 activity. This chapter will focus in greater depth on caveolae as structural microdomains of cAMP signalling in iPSC-CMs, both in the IMR and CDI cell lines, as it is an invaluable structural and functional feature of mature myocytes.

Caveolae are flask-shaped lipid rafts rich in caveolin (CAV) proteins, cholesterol and a variety of signalling molecules and receptors, including $\beta_2\text{AR}$ s in healthy cardiomyocytes. Clustering of these essential protein complexes within a defined domain increases efficiency of signal transduction; thus, caveolae are crucial structural and functional compartments. M β CD disrupts caveolae compartments of the membrane and has been shown to delocalise $\beta_2\text{AR}$ s (Nikolaev et al., 2006). Stimulation of $\beta_2\text{AR}$ after caveolae disruption leads to a change in the spatial characteristics of the downstream signalling pathway (Calaghan et al., 2008). In particular, cAMP no longer remains confined to the sarcolemma, but can be found diffused throughout the cytosol and is capable of targeting proteins of the SR and myofilaments. This consequently enhances the magnitude of $\beta_2\text{AR}$ -dependent response (Calaghan & White, 2006). It has been previously shown (and we have confirmed in the previous chapter) that iPSC-CMs do express both $\beta_1\text{AR}$ s and $\beta_2\text{AR}$ s, and their expression levels increase significantly during differentiation (Wu et al., 2015). Despite knowledge of CAV3 gene and protein expression in iPSC-CMs and hESC-CMs (Bosman et al., 2013; Jung et al., 2016; Haodi Wu et al., 2015), the presence of caveolae as functional components and their role in compartmentalising $\beta_2\text{AR}$ signalling is not fully understood.

Furthermore, an essential feature of the β_2 AR signal transduction system is the compartmentation of the cAMP molecules by phosphodiesterase (PDE) enzymes involved in the β AR signalling pathway.. PDEs are highly regulated enzymes which degrade cyclic nucleotides, thus can effectively and efficiently localise its activity; thus allowing specificity of response to a given stimulus (Epstein, 2017). Not only are they crucial for the general reduction of intracellular cAMP concentrations, they are responsible for creating and regulating cAMP gradients within the cell. This is thought to be achieved through the spatial confinement of PDEs as well as the functional coupling to individual receptors (Richter et al., 2008). In this way, local cAMP concentrations can be controlled in a stimulus-specific manner.

There are indications from previous studies that it is the cellular compartmentation of individual PDEs which determines their effect on intracellular cAMP rather than their expression levels. It has been reported that PDE2 and PDE4 modulate cAMP responses to β AR stimulation and PDE3 has a limited effect in rat ventricular myocytes (Mongillo et al., 2004, 2006). In addition, evidence suggests that in rat neonatal myocytes, PDE2 and PDE4 are functionally coupled to a pool of AC which is activated in response to β AR stimulation, thus regulating cAMP in defined cellular domains, whereas PDE3 may function outside of these domains (Zaccolo, 2006). In human hearts, PDE4 is an important contributor to β AR-dependent cAMP degradation activity, although it only contributes to 8% of all PDE expression (Richter et al., 2011). This may indicate, that despite its low expression level, it has a strong functional effect of contraction. Furthermore, PDE3 has been identified to be the dominantly expressed isoform in human cardiac tissue (Eschenhagen, 2013).

The differential subcellular localisations of the β AR subtypes may play a role in mediating differential responses in PDE activity; however, no such distinction between the two β AR subtypes with PDE activity has yet been investigated in iPSC-CMs. Investigation of PDE activity in response to β AR stimulation is of particular importance in iPSC-CMs, especially as it may provide an indication of cellular maturation or development. Previously it has been reported that iPSC-CMs do express β ARs,

although expression levels vary, and are also responsive to β AR agonist stimulation (Jung et al., 2016). Furthermore, PDE2, 3 and 4 gene expression can also be detected in iPSC-CMs. Gene expression evidence suggests that PDE2 levels are decreased throughout iPSC-CMs maturation, and PDE3 and PDE4 expression levels are gradually yet significantly increased (Wu et al., 2015). However, the functional activity of PDEs within caveolar membrane domains or their functional association to specific β AR subtype stimulation has yet to be investigated.

This chapter aims to understand the extent of caveolar microdomains in iPSC-CMs and its role in compartmentation of β_1 ARs or β_2 ARs and their associated cAMP signalling. Furthermore, this chapter will also focus on understanding whether PDE2, 3 or 4 are specifically associated with β_1 AR- or β_2 AR-dependent cAMP activity. Finally, this chapter will focus on identifying the association of the β AR-cAMP pools and PDE activity to caveolar microdomains.

5.2 METHODS

5.2.1 Cell culture

5.2.1.1 iPSC-CMs (CDI) plating and maintenance

One of the human iPSC-CMs lines used in this project were obtained from Cellular Dynamics International (CDIs). The 1.5mL cryopreserved single-cell suspension vials were thawed, seeded and cultured following the User Guide protocols as described in section 2.1.4. Cells were cultured for 10 days before experimental use in order to reach maximal stability. Results from the CDI cell line (D35) are presented in results sections 1-3.

5.2.1.2 iPSC-CMs (IMR) plating and maintenance

iPSCs of the IMR-90 cell line were differentiated until D15 and cultured to D30-D35 using the protocol described in Section 2.1.1. At D30-D35 cells were dissociated using the CDS method described previously. Results from the IMR cell line (D35) are presented in results sections 1-2 and 4-8.

5.2.2 Cell transfection for CAV3 overexpression

iPSC-CMs (IMR-90 cell line) were transfected at D30 with the CAV3-RFP plasmid in a similar way to the pTEV FRET sensor. Refer to section 5.2.6.2. CAV3-OX experiments were only conducted in the IMR cell line of iPSC-CMs (D35).

5.2.3 Removal of caveolae microdomains

Surface caveolae were removed from the cell membrane of both IMR and CDI iPSC-CMS cell lines by treating the cells with 1-2mM M β CD for 1h at 37°C. M β CD was prepared by dissolving it in either RB+ media for IMRs or Maintenance media for CDIs. After the cells were plated into MatTek dishes and

cultured to day 30 $\pm$ 5 days there were used to the FRET experiments. The previous media was removed and 1.5mL of the M β CD media was added. After exposure the M β CD media for one hour, cells were ready for experimentation.

5.2.4 Transmission Electron Microscopy

5.2.4.1 TEM protocol

For TEM preparations, 200k CDIs or IMRs iPSC-CMs were plated on 12-well plastic bottomed culture dishes, in a volume of 100 μ l of either iMaintenance media or RB+ respectively. This was topped up after 2h once the cells had adhered to the dish.

Culture medium was removed from cell culture dishes; cells were rinsed with PBS to remove any unadhered cells and debris, then fixed in 2.5% gluteraldehyde in cacodylate buffer. Further preparation was conducted by Ivan Diakonov as described in section 2.4.1

5.2.4.2 TEM analysis

From each TEM sample preparation, an “n” number of cells were processed. From each cell, 5-10 sections/frames were prepared. The total number of caveolae per frame were manually counted and used to calculate an average number of caveolae per cell. This was divided by the size of the frame to give the total number of caveolae per μ m. To test for statistical significance, a students T-test was conducted.

5.2.5 RT-QPCR

5.2.5.1 RNA extraction

CDIs' and IMRs were plated on gelatin-coated, plastic-bottom 6- or 12-well dishes and cultured until D35, and then prepared for RT-QPCR. RNA was extracted using the RNeasy Mini kit (Qiagen, CA, USA) from cultured hiPSC-CMs according to the manufacturer's guidelines as described in Section 2.3.1. The primer used are as follows:

Target Gene	Forward sequence	Reverse sequence
β_1 -Adrenoceptor	GACGCTCACCAACCTCTTCA	CACAGCTCGCAGAAGAAGGA
β_2 -Adrenoceptor	TTGCCTCTTCCATCGTGTCC	CCACCTGGCTAAGGTTCTGG
Caveolin 3	GCGGAAGGAGGTCTAAAGC	AGAGCGAAGGGCCATTGT
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Table 5. 1 Forward and reverse primer sequences used for RT-qPCR

5.2.5.2 RT-QPCR protocol

Gene expression profiles were investigated in CDI and IMR lines of iPSC-CMs at D30 of culture. iPSCs were plated and differentiated into cardiomyocytes as previously described. Once cells reached the age of 30 or 90 days, they were harvested and RNA was extracted as highlighted above. The Qiagen kit was used to perform RT-QPCR as described in section 2.3.2. All QPCR reactions were carried out in triplicates (Fig 5.1).

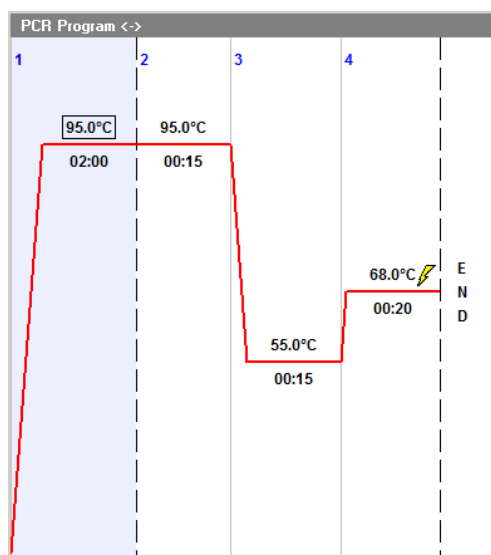


Figure 5. 1 The RT-QPCR program used, where stages 2-4 were repeated for 40 cycles.

5.2.5.3 RT-QPCR analysis

Prior to using the QPCR data, the melting curves were checked for the presence of a single peak through the cycle which rose above the threshold. This indicated that the amplified products were of a single species and off-targets products or contamination wasn't occurring. As these didn't occur, the data was used for analysis. The data was normalised to the housekeeping gene GAPDH. The fold differences were calculated through using the " $2^{-\Delta\Delta C_t}$ " method. Statistical significance was tested through the Students T-test.

5.2.6 FRET

5.2.6.1 iPSC-CMs (CDI) transfection with pTEV FRET sensor

The jetPRIME (Polyplus) transfection protocol was used to transfect CDI iPSC-CMs with the cAMP-specific FRET sensor pTEV. To prepare the transfection mixture, in a 1.5mL Eppendorf tube, 2µg of

pTEV plasmid was diluted in 50 μ L of room temperature jetPRIME buffer per MatTek dish to be transfected. This was then vortexed gently for 10sec. To this, 2 μ L of jetPRIME reagent was added then vortexed gently for 15s before incubating at room temperature for 10min. The 50 μ L transfection mixture was added to the centre of the MatTek culture dishes on top of the cells in and incubated for 4h at 37°C. The media in the dish was topped up with 1.5mL of CDI iMaintenance medium and incubated for a further 24-72h before use (Fig 5.2).

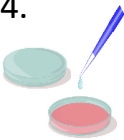
1.  Combine pTEV DNA + 50 μ L JetPrime Buffer
2.  Vortex on low speed for 10-15 seconds
3.  + 2 μ L JetPrime Reagent and vortex for 15 seconds
5.  Incubate for 10 minutes at room temperature
4.  Add transfection mixture to cells and incubate for 24-72 hours

Figure 5. 2 Transfection of iPSC-CMs (CDIs) for FRET experiments

5.2.6.2 iPSC-CMs (IMR) transfection with pTEV FRET sensor

iPSC-CMs were transfected with the cAMP-specific FRET sensor plasmid Turquoise-Epac1-Venus (pTEV), and/or CAV3 plasmid using Lipofectamine3000 kit. The manufacturer's guidelines were followed with optimization to incubation times. In brief, a mastermix was made based on the number of dishes being transfected where each dish would receive 50µL Opti-MEM (Thermo Fisher), 1.5µL Lipofectamine, 1.5µL Reagent P3000 and 1µg plasmid DNA. The plasmid DNA + Lipofectamine and Reagent P3000 were diluted in 25µL OptiMEM media in separate Eppendorf tubes. This was incubated for 5min at room temperature before combining the DNA and Lipofectamine reagents, making a total volume of 50µL. This was vortexed very gently for a few seconds to allow through mixing of the two reagents. This was then incubated at room temperature for 15min. The previous culture media was replaced with 1ml of fresh media before adding the transfection reagent. Cells were incubated at 37°C in the transfection reagent for 2-4 days and then used for experiments (Fig 5.3).

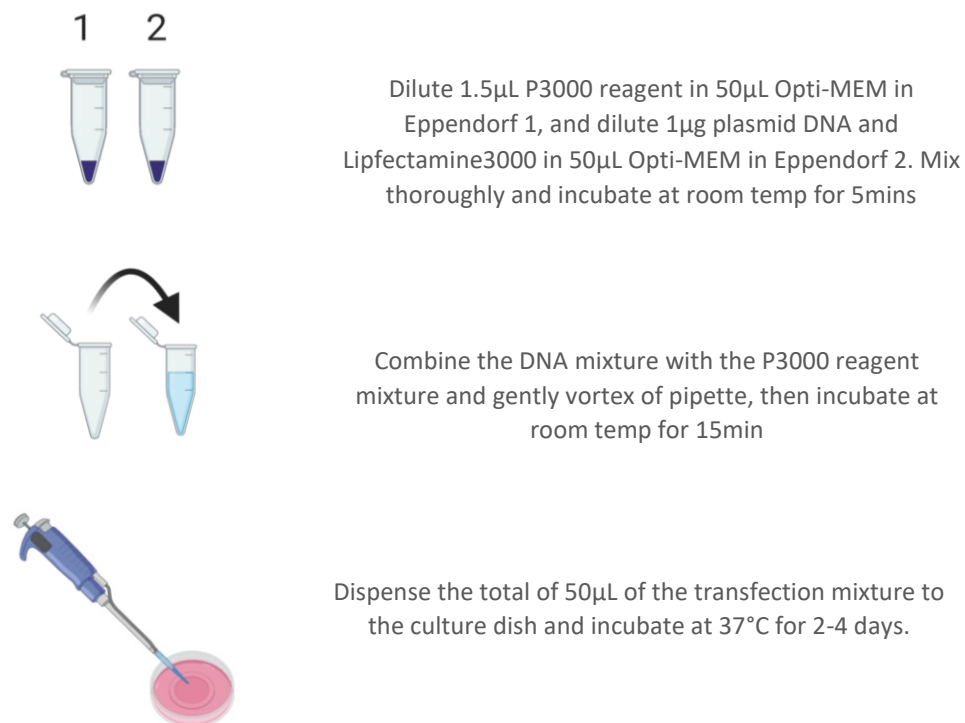


Figure 5. 3 Transfection of iPSC-CMs (IMRs) with CAV3 and pTEV FRET plasmid

5.2.6.3 Isoprenaline dose-response curves

Dose responses of both IMR and CDI iPSC-CMs cell lines for β AR stimulation with isoprenaline was conducted to assess the EC_{50} of both receptors. Concentrations of ISO were prepared in FRET buffer such that the final concentrations in the bath during the experiments were ranging from 0.1nM to 100nM. CGP20712A (CGP) (Sigma Aldrich, Poole UK) (100nM) was used for blocking β_1 AR and ICI (Tocris, UK) (50nM) was used for blocking β_2 AR prior to ISO stimulation. Upon reaching a plateau in the FRET response, cells were perfused with NKH477 (NKH) (Sigma Aldrich, Poole UK) (10 μ M) to fully stimulate all AC activity and achieve maximal cAMP release (Fig 5.4).

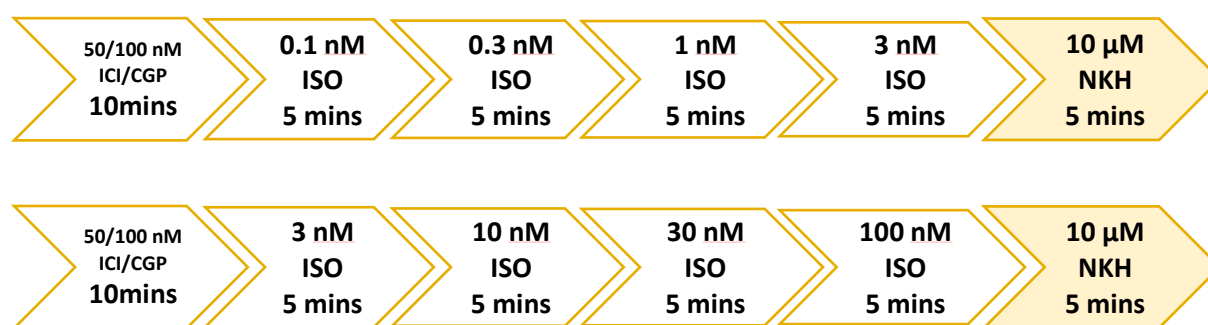


Figure 5. 4 Protocol for dose responses of β_1 AR and β_2 AR to isoprenaline used for FRET

Two protocols were used, the first where the final ISO concentrations ranged from 0.1nM to 3nM, the second where the final ISO concentrations were 3nM to 100nM. Both protocols were concluded with the final concentration of 10 μ M NKH.

5.2.6.4 Phosphodiesterase inhibition

The activity of β AR-dependent cAMP-degrading PDEs in hiPSC-CMs from IMR cell lines were conducted in this chapter. In these experiments, a single concentration of 30nM ISO was used. After β_1 AR or β_2 AR stimulation with 30nM ISO, PDE2, PDE3 and PDE4 were selective inhibited with 10 μ M EHNA, 10 μ M Cilostamide and 10 μ M Rolipram respectively which was prepared in FRET buffer. Once a plateau was

obtained during the FRET experiment, 100 μ M IBMX was added to the bath for non-selective inhibition of PDEs, followed by 10 μ M NKH for AC activation (Fig 5.5).

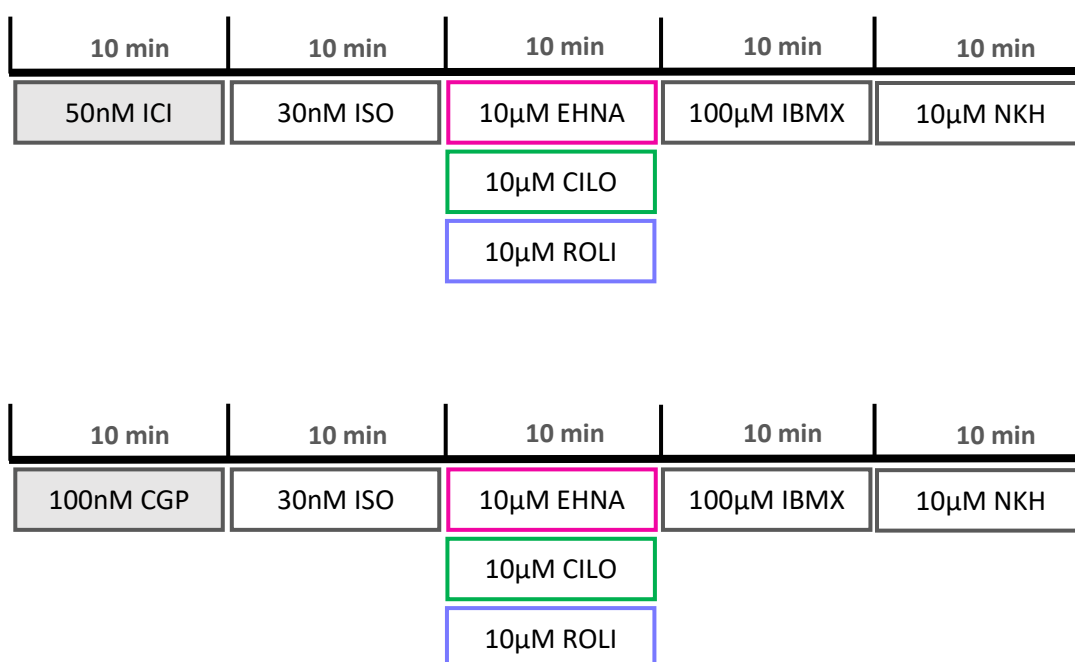
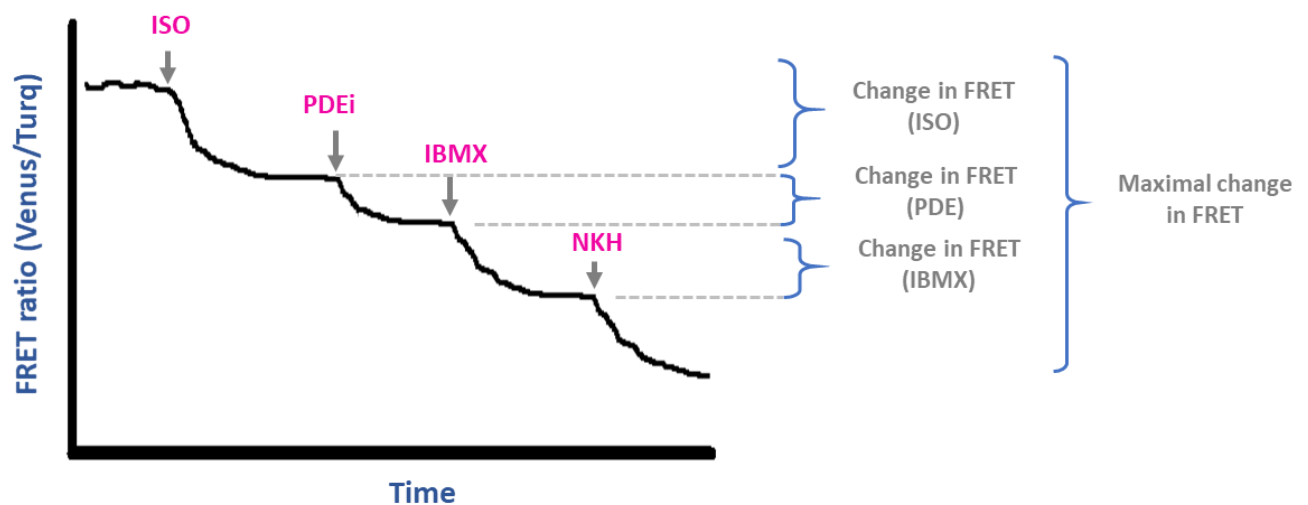


Figure 5. 5 β_1 AR or β_2 AR protocol for PDE activity assessment with the respective drug treatments of the stated final concentrations

5.2.6.5 FRET analysis

Analysis of the FRET results were conducted in Microsoft Excel. The ratio between the fluorescence recorded from the YFP channel and the CFP channel was calculated and plotted to obtain a regular YFP/CFP FRET trace. These results are shown as line graphs with the “FRET ratio (Venus/Turquoise)” plotted. From this the percentage change in the ratio from baseline to the drug treatment in question was calculated. The FRET response from ISO or the PDE inhibitor were normalised to the maximal drop recorded when the cells were treated with NKH. These results are shown as bar graphs plotted as “% FRET change Venus/Turquoise normalised to NKH”. In addition, the basal tone of the PDE is also reported. This was measured by calculating the change in FRET ratio from β AR stimulation with ISO to PDE drug treatment and is normalised to the maximal cAMP response from NKH treatment (Fig 5.6).



$$\% \text{ FRET change (normalised to NKH)} = \frac{\% \text{ Change in FRET (ISO)}}{\text{Maximal change in FRET}}$$

$$\% \text{ FRET change (Basal tone)} = \frac{\% \text{ Change in FRET (PDEi)}}{\text{Maximal change in FRET}}$$

Figure 5. 6 Diagram depicting calculations from the FRET traces

5.2.7 Statistical analysis

The statistical tests conducted has been described in each experimental method section. Generally, the statistical analysis used to analyse data obtained for experimental work was Students T-Test, which were performed using GraphPad Prism 5. The statistical significance was set at $p < 0.05$ and data has been reported with $\pm$ SEM (Standard error of the mean).

5.3 RESULTS

5.3.1 Analysis of caveolar compartments in two iPSC-CMs lines

To investigate caveolae compartments of the IMR and CDI human iPSC-CM cell lines, transmission electron microscopy was conducted on both the IMR and CDI iPSC-CMs cell lines. Analysis revealed differences in the number of surface membrane caveolae present in CDI versus IMR. CDI had approximately 0.37 ± 0.06 caveolae per μm membrane, however, IMR had a lower number of surface caveolae, 0.21 ± 0.04 caveolae per μm membrane ($p < 0.05$). In both CDI and IMR cell lines, treatment with M β CD significantly depleted caveolae content leading to only 0.05 ± 0.02 and 0.11 ± 0.02 caveolae per μm of membrane respectively ($p < 0.05$) (Fig 5.7 a-d). Upon assessment of gene expression levels of caveolin-3, both cell lines had similar levels of gene expression with no significant differences observed; 1.02 and 1.01 relative expression levels in CDI and IMR respectively (Fig 5.7e).

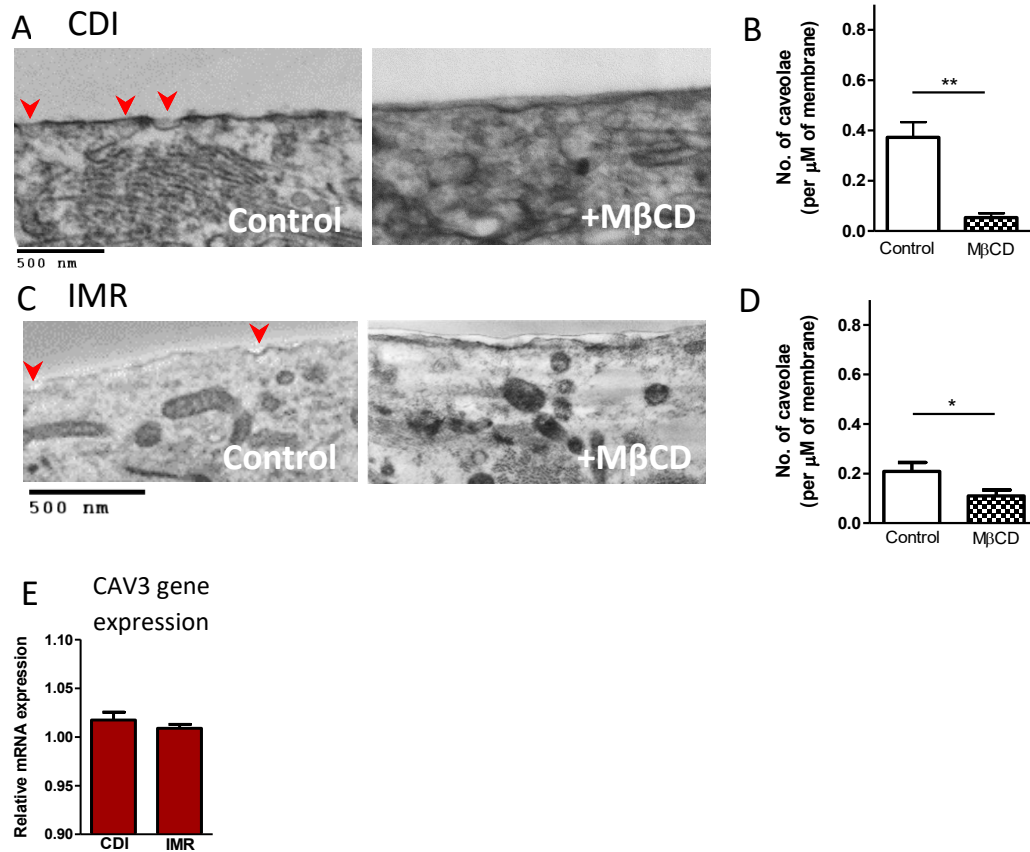


Figure 5. 7 Quantification of caveolae compartments and CAV3 gene expression in CDI and IMR

A) Representative TEM images showing surface caveolae (indicated with arrows) in control ($n=26/2$) and MβCD-treated ($n=10/2$) in CDI and B) its relative quantification. C) Representative TEM images showing surface caveolae (indicated with arrows) in control ($n=45/2$) and MβCD-treated ($n=37/2$) in IMR and D) its relative quantification. E) Quantification of the relative gene expression level of CAV3 in CDI and IMR. Scale bar represents $0.5\mu\text{M}$. Data are presented as mean $\pm$ SEM, $*p<0.05$, $**p<0.01$. $n=\text{cells/batches}$

5.3.2 β AR gene expression in two hiPSC-CMs lines and their relative cAMP activity

To understand the influence of caveolae compartments on β_1 AR and β_2 AR activity, firstly β AR expression and signalling properties were depicted in both the IMR and CDI iPSC-CMs cell lines.

To begin with, relative gene expression levels of β_1 AR and β_2 AR in CDI and IMR were calculated through RT-QPCR. CDI had a relative mRNA expression of 0.90 and 1.15 of β_1 AR and β_2 AR respectively; in comparison to IMR which had a relative expression of 1.01 and 1.05 of β_1 AR and β_2 AR. No significant differences in β_1 AR vs. β_2 AR in CDI or IMR were detected, nor were there any significant differences in CDI vs. IMR in β_1 AR or β_2 AR gene expression levels (Fig 5.8a).

β_2 AR-dependent cAMP activity was assessed in both CDI and IMR by investigating isoprenaline concentration-dependent responses by FRET microscopy. Both lines showed relatively small changes in FRET ratio for ISO concentrations of 0.1-1nM. The EC_{50} values of IMR and CDI were 3.3nM and 0.6nM respectively ($p < 0.0001$) (Fig 5.8b, c). These two iPSC-CMs cell lines show a significantly different EC_{50} values, where the CDI cell line shows significantly greater sensitivity to β_2 AR stimulation with isoprenaline than the IMR line ($p < 0.05$). There is no statistically significant difference in the maximal output of the β_2 AR response to ISO of the CDI and IMR cell lines ($p = 0.06$).

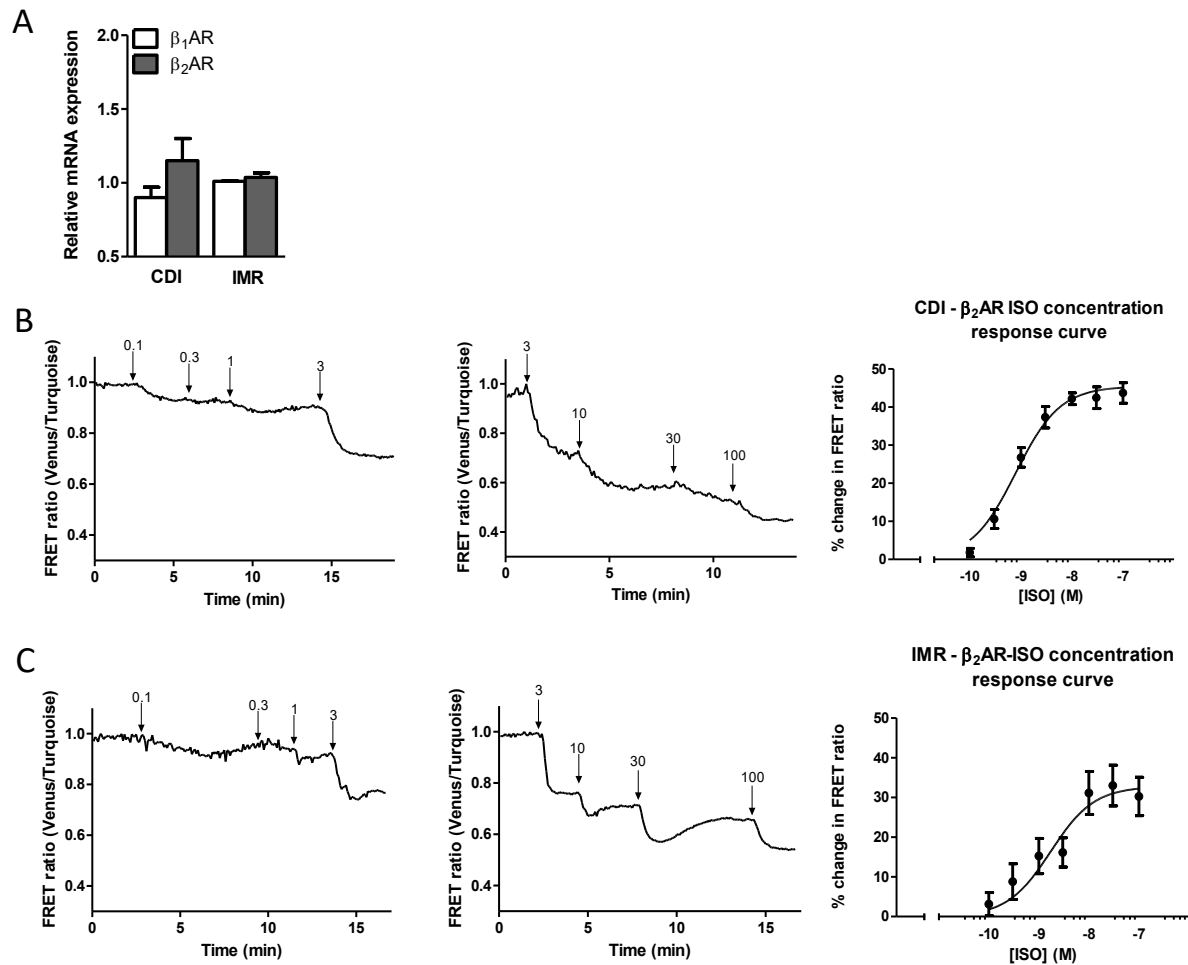


Figure 5. 8 β_1 AR and β_2 AR response to stimulation with ISO in CDI's in control and M β CD treated cells

A) Gene expression of β_1 AR and β_2 AR in IMR (n=9) and CDI (n=4). B) Representative FRET traces and concentration-response curve of CDI in response to β_2 AR stimulation with ISO concentrations ranging from 0.1nM to 100nM in the presence of CGP (n=9/3). C) Representative FRET traces and dose response curve of IMR in response to β_2 AR stimulation with ISO concentrations ranging from 0.1nM to 100nM in the presence of CGP (n=7/5). Data are presented as mean $\pm$ SEM. n= cells/batches

5.3.3 Caveolar compartmentation of β AR-dependent cAMP activity in CDIs

The CDI cell line shows a greater number of caveolae microdomains and displays greater sensitivity to β_2 AR stimulation with isoprenaline. The role of caveolae in compartmentation of the β_2 AR signalling was explored next. FRET microscopy was employed to investigating β_1 AR and β_2 AR cAMP output upon receptor stimulation with ISO in the presence or absence of caveolae. Cells were treated with M β CD for the depletion of caveolae.

Upon stimulation of β_1 AR, a $2.4 \pm 1.1\%$ change in FRET ratio was detected, and a significantly greater change of $39.3 \pm 2.6\%$ after AC stimulation with NKH ($p < 0.05$). Removal of caveolae with M β CD resulted in a $9.0 \pm 2.5\%$ change in FRET ratio upon β_1 AR stimulation and $31.2 \pm 6.8\%$ after NKH treatment. There were no significant differences found in β_1 AR-dependent cAMP activity after caveolae depletion.

β_2 AR-dependent cAMP activity was then assessed. β_2 AR stimulation led to a $3.9 \pm 2.2\%$ change in FRET ratio and a $31.3 \pm 4.5\%$ change upon AC activation with NKH. After the depletion of caveolae with M β CD, there was a $29.3 \pm 4.5\%$ change in FRET with ISO and a $36.2 \pm 3.2\%$ change in FRET with NKH treatment. There was a significantly greater amount of cAMP activity after β_2 AR, but not β_1 AR, stimulation after the depletion of caveolae ($p < 0.05$) (Fig 5.9).

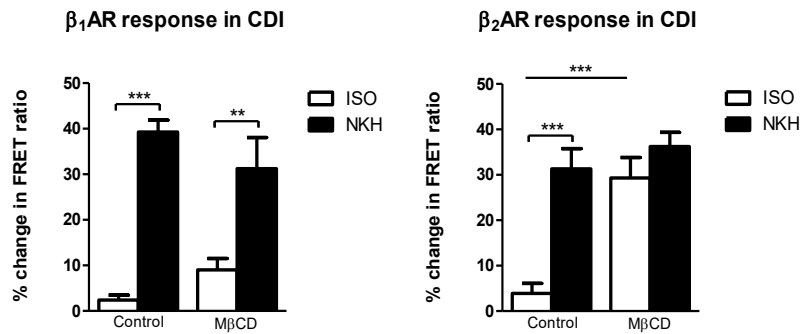


Figure 5.9 β_1 AR and β_2 AR response to stimulation with ISO in CDI's in control and MβCD treated cells

The association of β_1 AR- ($n=19/3$) and β_2 AR-cAMP ($n=18/5$) activity with caveolae was assessed in CDI's with isoprenaline in the presence of ICI or CGP respectively, with subsequent activation of AC with NKH to achieve maximal cAMP production. MβCD was used to deplete caveolae in order to depict the role of caveolae in compartmentalising the β_1 AR ($n=6/2$) and β_2 AR ($n=9/3$) response. Data are presented as mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

5.3.4 Caveolar compartmentation of β AR-dependent cAMP activity in IMRs

Similar to the previous experiment, the influence of caveolae on the compartmentation of the β_1 and β_2 adrenergic receptors and their associated cAMP pools was investigated. FRET microscopy was used to record cAMP output upon receptor stimulation with ISO was assessed in the presence or absence of caveolae MβCD. β_1 AR stimulation lead to a $21.6 \pm 1.8\%$ change in FRET ratio, and a $15.2 \pm 2.0\%$ change in FRET after caveolae depletion (Fig 5.10 a, e). Upon β_2 AR stimulation, there was a $47.7 \pm 4.5\%$ change in FRET, and a $63.14 \pm 7.7\%$ change after caveolae depletion (Fig 5.10 b, e). Firstly, a significantly greater amount of β_2 AR-cAMP activity than β_1 AR-cAMP activity was observed in the IMR cell line ($p<0.05$). This is unlike the CDI line, where the β_1 AR- and β_2 AR-cAMP responses were similar. Removal of caveolae with MβCD had no effect on β_1 AR-cAMP output, and a small, but not statistically significant, increase in β_2 AR-cAMP output, although statistical significance was not obtained. When CAV3 was overexpressed, there was a significantly decreased amount of cytosolic cAMP upon β_2 AR stimulation detected, $13.3 \pm 5.5\%$ FRET change ($p<0.05$), but no difference was detected for β_1 AR stimulation, $13.0 \pm 2.1\%$ FRET change (Fig 5.10).

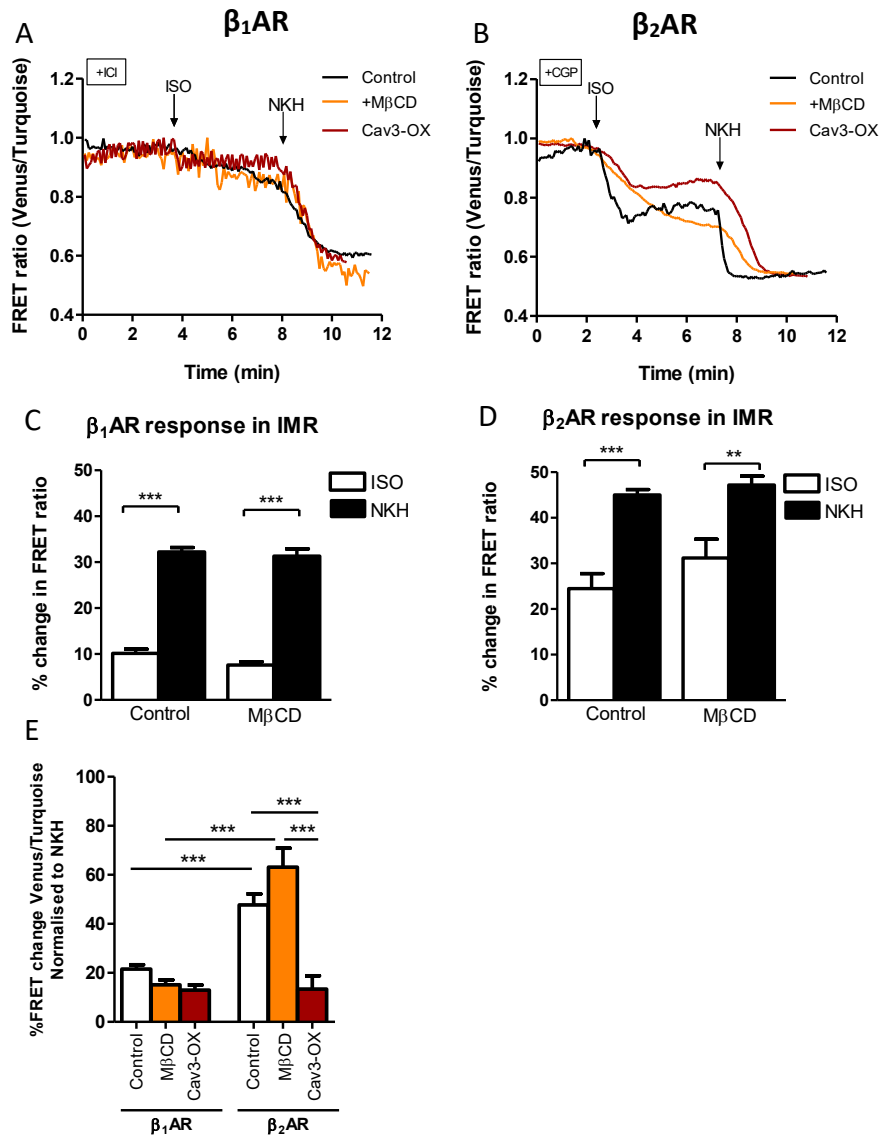


Figure 5. 10 β_2 AR compartmentalise within caveolae microdomains which localise cAMP

A) Representative FRET traces in control, M β CD-treated and CAV3-OX cells. β_1 AR stimulation with isoprenaline in the presence of β_2 AR blocker and subsequent AC stimulation with NKH477. B) Representative FRET traces in control, M β CD-treated and CAV3-OX iPSC-CMs upon β_2 AR stimulation with isoprenaline in the presence of β_1 AR blocker and subsequent adenylyl cyclase stimulation with NKH477. C) β_1 AR and β_2 AR response to stimulation with ISO in IMR's in control and M β CD treated cells D) The percentage change in FRET ratio upon β_1 AR stimulation in control (n=97/11), +M β CD (n=62/11) and CAV3-OX (n=6/2); and β_2 AR stimulation in control (n=38/4), +M β CD (n=28/3) and CAV3-OX (n=9/2). Percentage change was calculated and normalised to maximal cAMP released by NKH stimulation. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * p <0.05, *** p <0.001. n=cells/batches

5.3.5 β_1 AR and β_2 AR-cAMP dependent PDE2 activity in iPSC-CMs (IMR line)

In the following sections and in the next chapter, the activity of PDEs 2, 3 and 4 will be assessed and their functional association to either of the β AR subtype-specific cAMP pools or to caveolae itself in the IMR cell line of iPSC-CMs as the CDI cell line shows greater caveolar development and a relatively small β_2 AR activity. Hence, to assess maturation in the next chapter, the IMR cell line was more suitable. Secondly, as they are produced in-house, they can be produced in large batches, making experiments cost-effective.

To characterise the role of PDE2 in IMR, its specific inhibitor EHNA was used to block enzyme activity, which selectively blocks PDE2 at micromolar concentrations, and only partially inhibits other PDEs at concentrations greater than 100 μ M. To discover the role of PDE2 in cAMP degradation, its FRET response was calculated relative to total cAMP activity, using NKH for maximal cAMP output.

When the response to drug treatment relative to total cAMP by treatment with NKH was analysed, a $21.6 \pm 1.8\%$ of total β_1 AR-dependent cAMP output was observed from isoprenaline treatment and $26.9 \pm \%$ after PDE2 inhibition, and a significant increase from $16.1 \pm 2.1\%$ to $37.0 \pm 8.5\%$ upon isoprenaline and EHNA treatment respectively after caveolae depletion ($p < 0.05$). A β_2 AR-dependent response to isoprenaline and EHNA was $46.3 \pm 4.4\%$ to $51.0 \pm 5.0\%$ cAMP output in control conditions. After the removal of caveolae, a significant increase was noted, from $63.1 \pm 7.7\%$ to $96.4 \pm 2.2\%$ upon isoprenaline and EHNA treatment respectively ($p < 0.05$) (Fig 5.11c).

The basal tone of PDE2 was extracted from this experiment to focus on PDE2 activity alone. PDE2 activity on β_1 AR-dependent cAMP was only $7.1 \pm 1.6\%$ of the total FRET change. Upon depletion of caveolae, there was a slightly reduced influence of PDE2 of $3.7 \pm 3.0\%$, although this was not significantly different. The effect of PDE2 on β_2 AR-dependent cAMP was $16.8 \pm 4.4\%$ of total activity. After the removal of caveolae, this response was almost undetectable, unfortunately due to the high response to isoprenaline stimulation (Fig 5.11d).

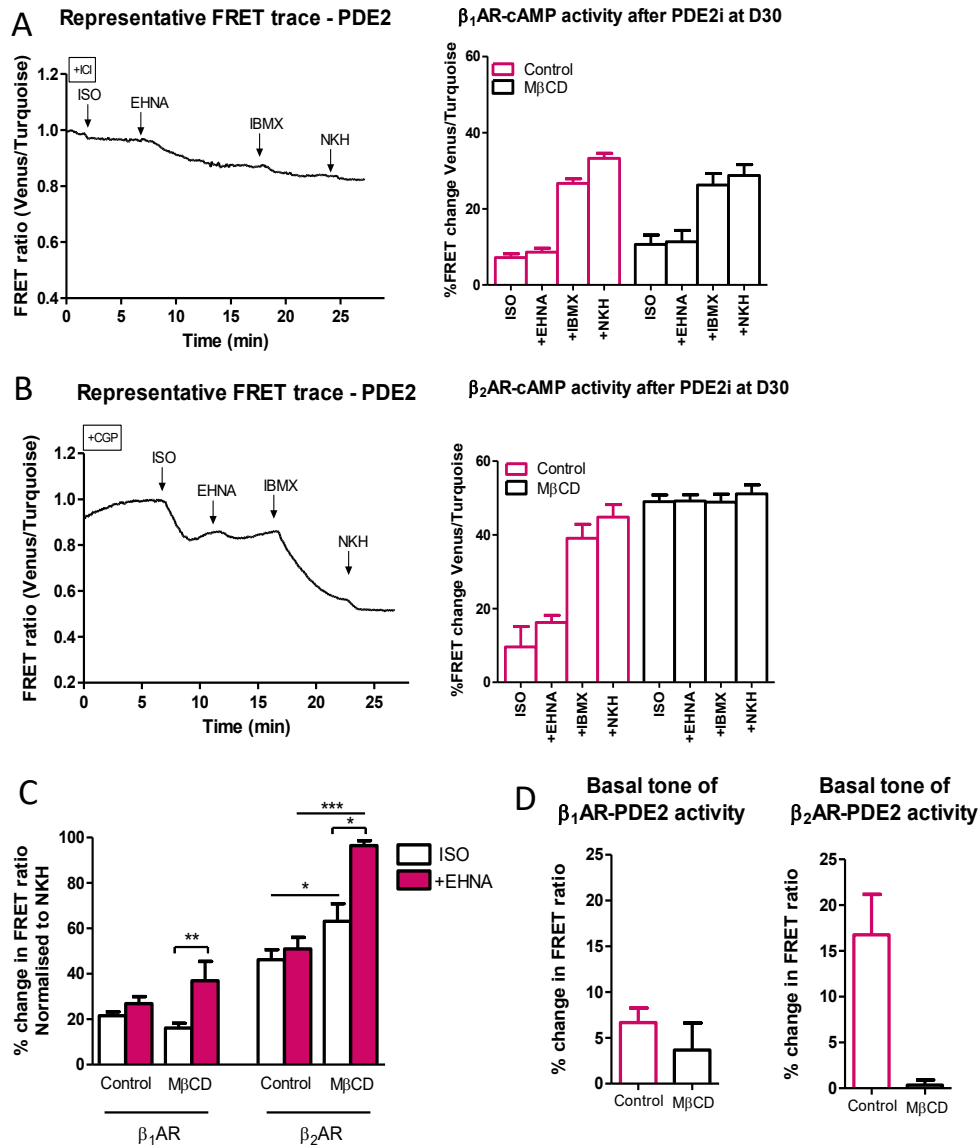


Figure 5. 11 β_1 AR and β_2 AR dependent PDE2 activity in D30 iPSC-CMs (IMR cell line)

The association of β_1 AR- and β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D30 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE2 with EHNA. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β_1 AR stimulation and quantified percentage FRET change in control ($n=26/5$) and M β CD ($n=15/3$). B) Representative FRET trace upon β_2 AR stimulation and quantified percentage FRET change in control ($n=7/2$) and M β CD ($n=4/2$). C) Comparison of β_1 AR- and β_2 AR response to PDE2 inhibition in control and M β CD treated cells where the response is normalised to the effect of IBMX or NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and M β CD treated cells. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

5.3.6 β_1 AR and β_2 AR-cAMP dependent PDE3 activity in iPSC-CMs (IMR line)

Next, PDE3 activity was assessed in the IMR cell line of iPSC-CMs. The role of PDE3 was also characterised using its specific inhibitor Cilostamide to block enzyme activity. Unexpectedly, unlike the response for EHNA, when Cilostamide was applied for PDE3 inhibition, FRET ratio increased, indicating a reduction in cytosolic cAMP. This was seen in both β_1 AR and β_2 AR experiments. When IBMX and NKH were applied, the FRET ratio decreased indicating an increase in cAMP.

PDE3 inhibition after β_1 AR stimulation with isoprenaline led to a significant decrease in cAMP output from $21.6 \pm 1.8\%$ with isoprenaline to $2.1 \pm 3.2\%$ ($p < 0.05$). Similarly, after the removal of caveolae by treating with M β CD, a change from $16.1 \pm 2.1\%$ to $10.0 \pm 5.6\%$ was observed with isoprenaline and cilostamide respectively. A similar decreasing trend in FRET was observed upon PDE3 inhibition after β_2 AR stimulation. A significant decrease from $46.3 \pm 4.4\%$ upon isoprenaline stimulation to $32.0 \pm 5.3\%$ after cilostamide administration was observed ($p < 0.05$). Similarly, after M β CD treatment, a drop from $63.1 \pm 7.7\%$ to $49.1 \pm 2.7\%$ was observed respectively (Fig 5.12c).

Upon assessment of the tone of PDE3 itself, interestingly its inhibition had an opposing response and led to a decrease in cytosolic cAMP. PDE3 activity on β_1 AR-dependent cAMP led to a $-13.1 \pm 5.0\%$ FRET change, and removal of caveolae resulted in a $-33.4 \pm 6.3\%$ FRET change. PDE3 activity on β_2 AR-dependent cAMP led to a $-14.5 \pm 8.0\%$ FRET change with a similar response after the removal of caveolae (Fig 5.12d).

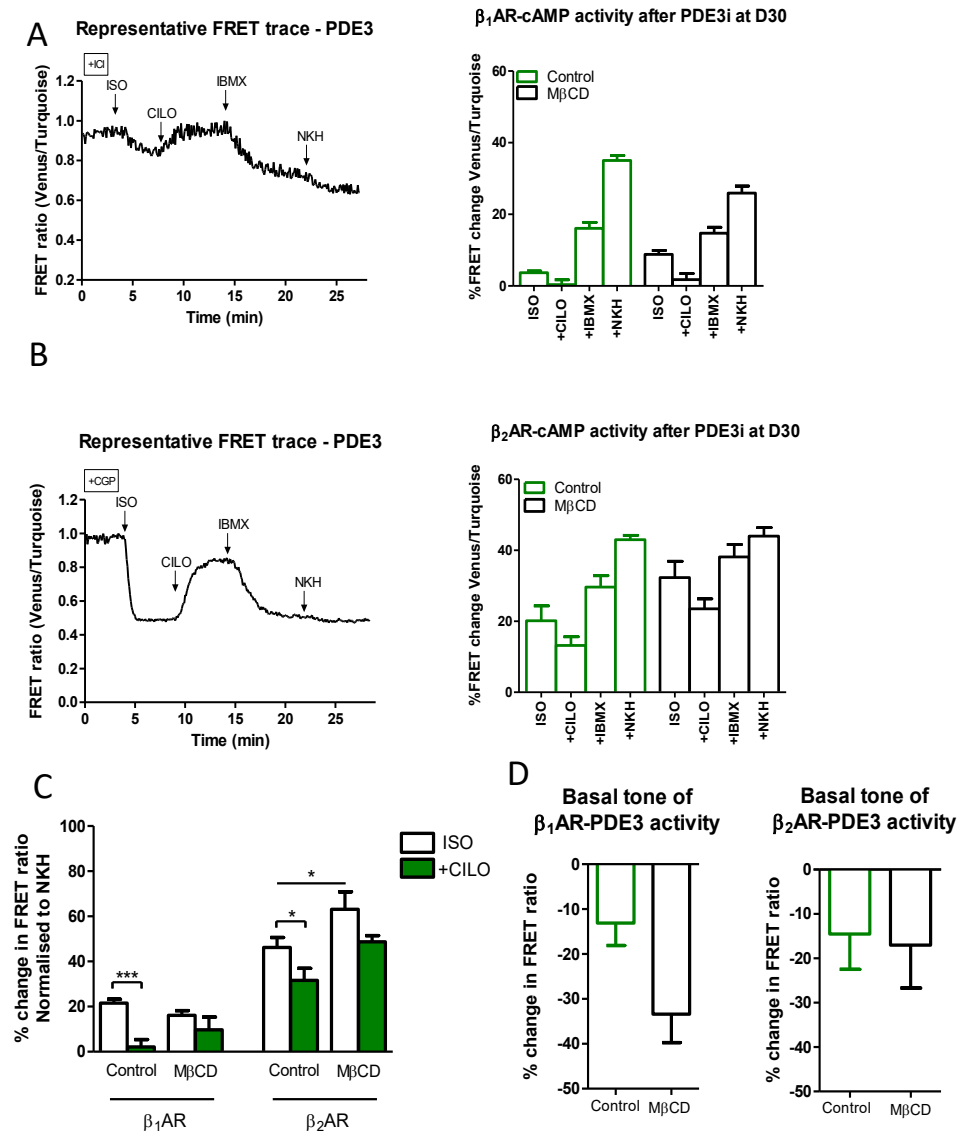


Figure 5. 12 β_1 AR and β_2 AR dependent PDE3 activity in D30 iPSC-CMs (IMR cell line)

The association of β_1 AR- and β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D30 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE3 with Cilostamide. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β_1 AR stimulation and quantified percentage FRET change in control ($n=33/2$) and M β CD ($n=25/2$). B) Representative FRET trace upon β_2 AR stimulation and quantified percentage FRET change in control ($n=22/7$) and M β CD ($n=12/3$). C) Comparison of β_1 AR- and β_2 AR response to PDE3 inhibition in control and M β CD treated cells where the response is normalised to the effect of IBMX or NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and M β CD treated cells. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

5.3.7 β_1 AR and β_2 AR-cAMP dependent PDE4 activity in iPSC-CMs (IMR line)

Lastly, the role of PDE4 in IMR cell line of iPSC-CMs was then assessed. Rolipram was used as a PDE4-specific inhibitor to block its enzymatic activity. To understand the role PDE4 plays in cAMP degradation relative to total cAMP using NKH for AC activation to obtain maximal cAMP output from the cell. The response relative to total cAMP by NKH treatment was analysed.

Under control conditions, a significant increase in β_1 AR-dependent cAMP output after rolipram treatment was observed, from $21.5 \pm 1.8\%$ to $74.7 \pm 3.5\%$ ($p < 0.05$). Similarly, after M β CD treatment, change from $16.1 \pm 2.1\%$ to $62.4 \pm 6.2\%$ was observed respectively. A β_2 AR-dependent response in control conditions also showed a significant increase in FRET change from $46.3 \pm 4.4\%$ after isoprenaline addition to $71.1 \pm 4.3\%$ after rolipram addition, and after caveolae removal with M β CD an increase from $63.1 \pm 7.7\%$ to $90.0 \pm 8.0\%$ FRET ratio was observed after caveolae removal ($p < 0.05$) (Fig 5.13c).

When assessing the basal tone of PDE4, it was found that PDE4 activity on β_1 AR-dependent cAMP was $30.0 \pm 5.1\%$ of total cAMP output. Upon depletion of caveolae, there was a slightly higher influence of PDE4, although this was not significantly different. The effect of PDE4 on β_2 AR-dependent cAMP was $17.1 \pm 5.2\%$ of total activity and again, the removal of caveolae did not significantly alter the response (Fig 5.13d).

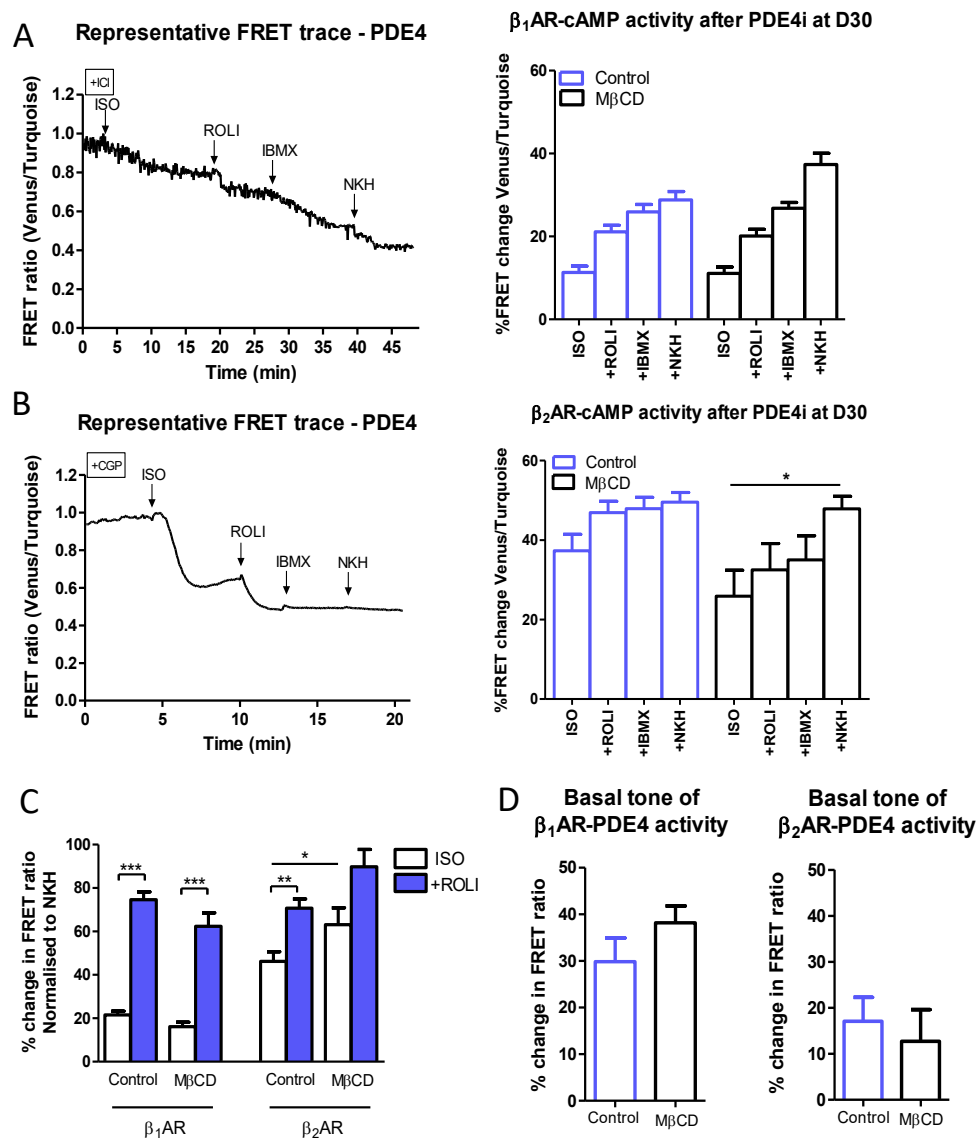


Figure 5. 13 β₁AR and β₂AR dependent PDE4 activity in D30 iPSC-CMs (IMR cell line)

The association of β₁AR- and β₂AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D30 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE4 with Rolipram. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β₁AR stimulation and quantified percentage FRET change in control (n=38/6) and MβCD (n=28/4). B) Representative FRET trace upon β₂AR stimulation and quantified percentage FRET change in control (n=29/3) and MβCD (n=17/3). C) Comparison of β₁AR- and β₂AR response to PDE4 inhibition in control and MβCD treated cells where the response is normalised to the effect of IBMX or NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and MβCD treated cells. Data are presented as mean ± SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. *p<0.05, **p<0.01, ***p<0.001. n=cells/batches

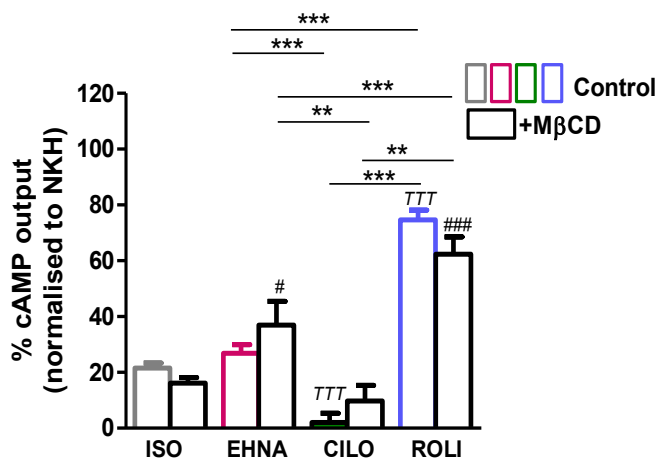
5.3.8 Comparing β AR-cAMP dependent PDE2, PDE3 and PDE4 activity in iPSC-CMs

An overall comparison of PDE2, PDE3, and PDE4 activity against β_1 AR- and β_2 AR-dependent cAMP in the IMR cell line of iPSC-CMs at D30 of differentiation is summarised in the graphs below. In comparison to the amount of cAMP produced by β_1 AR stimulation by isoprenaline in control, there was no significant difference with EHNA to inhibit PDE2. Inhibition of PDE3 significantly decreased cAMP output ($p < 0.05$), while PDE4 inhibition significantly increased cAMP output ($p < 0.05$). The removal of caveolae significantly increased cAMP output in the absence of PDE inhibitors ($p < 0.05$), but no significant influence of PDE2, 3, or 4 activity was detected on β_1 AR-dependent cAMP when caveolae components were removed (Fig 5.14).

When investigating β_2 AR-cAMP dependent PDE activity, no statistically significant difference in cAMP output was observed. Interestingly, the removal of caveolae significantly augmented PDE2 and PDE4 activity resulting in a near maximal response being obtained ($p < 0.05$).

A

β_1 AR-dependent PDE activity at D30



B

β_2 AR-dependent PDE activity at D30

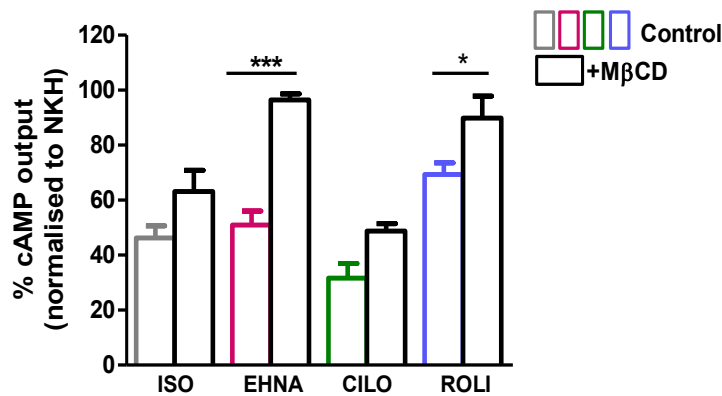


Figure 5. 14 β AR-dependent PDE activity in D30 iPSC-CMs (IMR) in control and MβCD treated cells

A) The association of β_1 AR- and B) β_2 AR-cAMP-degrading PDE activity with caveolae was assessed in iPSC-CMs at D30 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE2, 3 or 4 with EHNA, Cilostamide or Rolipram. The percentage change in FRET ratio was normalised to IBMX, or maximal cAMP release upon AC activation by NKH. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

5.4 DISCUSSION

This chapter aimed to understand the influence of caveolar compartmentation on β_1 AR- and β_2 AR-cAMP signalling, which was tested in two iPSC-CMs lines, and also to depict whether PDEs 2, 3, and 4 are associated with specific β AR-cAMP pools of iPSC-CMs.

Although both the IMR and CDI iPSC-CMs cell lines show similar levels of CAV3 gene expression, the CDI cell line possesses a greater density of caveolae. While a comparison with human adult myocytes has not been conducted in this study, previous reports indicate that CAV3 expression and caveolae structures in iPSC-CMs are relatively lower than that of mature adult cardiomyocytes where adult myocytes have a density of around 3 caveolae/ μm (Bosman et al., 2013; Egorov et al., 2019). Bosman and colleagues show that CAV3 is present over the whole surface of human adult atrial and ventricular myocytes, and although human foetal myocytes do display CAV3 protein to a certain extent, it is dispersed throughout the cell (Bosman et al., 2013; Jung et al., 2016). This may explain the findings of this study, although expression of CAV3 is detected in IMR and CDI cell lines, the localisation of CAV3 protein within the cell should be investigated, which may explain the differences in caveolae complexes seen. Interestingly, CAV3 increases dramatically with increasing cell culture time in hESC-CMs as well as iPSC-CMs (Bosman et al., 2013; Jung et al., 2016). Furthermore, this study confirms that treating iPSC-CMs with M β CD successfully removes a significant proportion of caveolae structures.

The β AR system is a crucial signalling pathway for cardiomyocytes to conduct various physiological and pathophysiological outputs in response to stimulation by the autonomic nervous system hormones. Interest in this signalling pathway is increasing in hPSC-CMs. In this report, the expression of the β AR receptor subtypes is evident in both iPSC-CM cell lines which displayed similar levels of β_1 AR and β_2 AR gene expression at Day 30 of differentiation. This is supported by previous literature which also demonstrate the expression of these receptors in iPSC-CMs and hESC-CMs (H Wu et al., 2014; Yan et al., 2011). In addition, they show that the expression of β_1 AR and β_2 AR in iPSC-CMs

increases throughout differentiation. They show that β_2 AR is expressed at maximal levels by day 12 of differentiation and expression remains constant until day 60; however, β_1 AR expression is only significantly increased at D30 of culture and continues to increase until day 60 (H Wu et al., 2014).

The responsiveness of the two iPSC-CM cell lines to β_2 AR stimulation was essential to understand the functional dynamics of the cells. Previously, studies have investigated β AR activity through measuring chronotropic responses and report functionally active β ARs with marked concentration-dependent positive inotropic and chronotropic effects (Germanguz et al., 2011; Haodi Wu et al., 2015). Other studies depicted that β_2 AR mediate the vast majority of the β AR activity at D30 (Jung et al., 2016) ; or that iPSC-CMs at D30 only respond to β_2 AR activation (Haodi Wu et al., 2015). Therefore, in this study, the functional activity of β_2 AR was initially assessed as this is known to be the dominant receptor subtype. cAMP signalling was measured in real-time using FRET microscopy upon receptor stimulation with isoprenaline in a dose-dependent manner. Both iPSC-CM lines showed an increased response upon increasing isoprenaline concentration, although they displayed variable sensitivities. This may indicate differences in the signal transduction pathway or receptor compartmentation.

A characteristic of mature healthy myocytes is the compartmentalisation of the β_2 AR-cAMP signalling which is mediated by the physical restriction of the receptor to caveolae. Previously, one study reported that compartmentation of β AR signalling occurs in iPSC-CMs post-D60 in culture as treatment with M β CD scattered clustered CAV3 structures and increased ISO-induced phospho-PLM and phosphor-Tnl compared to ISO without M β CD (Jung et al., 2016). To study the compartmentation of β_2 ARs to caveolar microdomains in this report, the effect of removing caveolae by treatment with M β CD on cAMP output was assessed using FRET microscopy. Results showed increased β_2 AR signalling after M β CD treatment but not β_1 AR signalling in both iPSC-CMs lines, indicating compartmentation of the β_2 ARs within the caveolae microdomains. This is supported further by the reduction in β_2 AR-cAMP activity in cells where the CAV3 gene was over expressed, indicating that β_2 AR compartmentalise to caveolae microdomains, hence cAMP signalling is restricted.

PDEs are essential components of the β_2 AR signalling pathway as they hydrolyse the phosphodiester bond of cyclic nucleotides and contribute to the distribution, duration and amplitude of signalling; therefore, the downstream PDE activity in iPSC-CMs is an important aspect to assess. Previously the function of PDE2 and PDE3 has been assessed in iPSC-CMs using ELISA-based cAMP assay assessments and have both been associated with regulating β AR dependent cAMP activity (Jung et al., 2016). However, previous studies do not address β AR subtype-specific PDE activity, nor was PDE4 (a major contributor to cAMP handling in adult myocytes) assessed.

In this study the real-time activity of PDE2, 3 and 4 on β AR subtype-specific cAMP pools were assessed using FRET microscopy. As discussed earlier, PDE2 is a dual-substrate enzyme with hydrolyses both cAMP and cGMP, and is stimulated by cGMP; therefore, making PDE2 an important negative feedback regulator of cAMP with cGMP increases. Previous literature suggests that PDE2A subtypes are located within the cell cytosol and cellular membranes (Guellich et al., 2014). PDE2 activity has been demonstrated to be relatively low in comparison to other cardiac PDEs in humans (Fischmeister et al., 2005), which supports the findings from this study as PDE2 inhibition did not result in any significant differences in cAMP output and non-selective inhibition of all other PDEs dramatically increased cAMP output, suggesting that other PDEs may be playing a greater role. Furthermore, it has been demonstrated that PDE2 gene expression levels are significantly reduced in iPSC when differentiated into iPSC-CMs, and become significantly elevated in disease conditions (Haodi Wu et al., 2015).

Furthermore, this study highlights that iPSC-CMs do not display any such specificity towards β_1 AR or β_2 AR cAMP pools as PDE2 inhibition did not augment either of these cAMP pools upon receptor stimulation. A previous study indicates that the β_1 AR microdomain is controlled by PDE2, and in disease PDE2 are redistributed from β_1 AR to β_2 AR membrane domains and so there is an increase in global β_1 AR-associated cAMP pool and a decrease in β_2 AR-cAMP (Perera et al., 2015). Another recent study demonstrates using FRET-based imaging that cAMP levels in neonatal rat cardiomyocytes are regulated by PDE2 activity downstream of β_3 AR (Stangherlin et al., 2011). Although β_3 AR expression

in iPSC-CMs is low (Jung et al., 2016) , and β_3 AR studies are beyond the scope of this study, there may be a raise in interest in future studies.

Interestingly, the disruption of caveolae compartments, enhanced PDE2 activity irrespective of β AR subtype. This may indicate that PDE2 functions not in the caveolar domains but in the cell periphery because significant PDE2 activity was only detected once the physical compartmentation of the cell membrane was disrupted and cAMP pools were dispersed throughout the cell. Moreover, when cells were treated with the non-selective PDE inhibitor, there was greater cAMP output, again indicating that PDEs other than PDE2 are majorly accountable for cAMP degradation in iPSC-CMs.

PDE3 is also an important regulator of cAMP in myocytes, therefore its activity was assessed in this study in iPSC-CMs. Like PDE2, PDE3 hydrolyses both cAMP and cGMP, but is inhibited by cGMP (Shakur et al., 2000). PDE3A and PDE3B subtypes are both expressed in the cardiomyocyte and their isoforms are found in both the particulate and cytosolic fractions (J Beavo & Houslay, 1990; Hambleton et al., 2005b; Wechsler et al., 2002b). PDE3 has been identified as a major cardiac PDE in adult myocytes, and the PDE3B subtype is shown to be expressed at higher levels than PDE3A (C Lugnier et al., 1993). PDE3 inhibition in adult myocytes leads to direct positive inotropic effects (C Lugnier et al., 1993). Upon assessment of PDE3 activity in iPSC-CMs, it was found that inhibition of PDE3 actively reduced cytosolic cAMP. This response was observed upon both β_1 AR and β_2 AR stimulation, and also upon the disruption of membrane microdomains, and the basal tone of PDE3 remained relatively similar between these different conditions. The reasons for this are not fully conclusive from this study. However, the answer may lie in the cyclic nucleotide cross-talk. The incorporation of different subtypes of PDEs, possessing distinct characteristics and regulatory properties, contribute to cyclic nucleotide signalling compartmentation within signalosomes in specific fashions. Therefore, this allows integration and crosstalk between the cyclic nucleotide signalling networks (Dodge-Kafka et al., 2010; Pavlaki & Nikolaev, 2018; Stangherlin et al., 2011). Many studies have highlighted the role that PDEs play in the crosstalk between the cAMP/cGMP signalling pathway (Ahmad et al., 2015; C. Y. Zhao,

2017). There is previous indication that different sensitivities to cGMP of cAMP-hydrolysing PDEs, cGMP (at concentrations less than 50nM) exclusively inhibits PDE. However, at greater cGMP concentrations, PDE2 and PDE1 become activated (Zaccolo & Movsesian, 2007). Although the concentration of cGMP was not identified, it would be an important factor for considering the decrease in cAMP observed upon PDE3 inhibition.

PDE4 is the largest family of PDEs and a major cAMP-hydrolysing enzyme. PDE4D subtypes are found to be localised in the perinuclear region, sarcomeric region and have also shown to associate directly with β ARs (Dodge-Kafka et al., 2005; Richter et al., 2008a, 2013). Catecholamine-induced activation of β_1 ARs and β_2 ARs activates different signalling pathways, for example β_1 AR activation results in a greater increase in heart rate and contractility than β_2 AR activation. PDE4 isoforms regulate β AR signalling via direct interaction with the receptors themselves (Richter et al., 2008a, 2013). In this study we find that PDE4 has the greatest role in cAMP degradation in iPSC-CMs as its inactivation led to the greatest cAMP output in comparison to PDE2 or PDE3. The role of caveolae microdomains in compartmentalising PDE4 activity was also assessed in this study. Upon caveolae depletion, similar increases in cytosolic cAMP were detected from control. This may suggest that PDE4 are not localised to membrane domains. Furthermore, it showed no bias towards β_1 AR- or β_2 AR-dependent stimulation. This could be explained by the understanding that different PDE4 subtypes are found with in discrete subcellular domains. For example, one study shows that PDE4B is localized in the plasma membrane, whereas PDE4D is mainly distributed in the cytosol (Blackman et al., 2011). Therefore, further studies are required to develop a deeper understanding of the various PDE4 subtypes within iPSC-CMs.

Limitations and future work

In this study a cytosolic FRET sensor was utilised. The pTEV sensor made use of optimised fluorophores, turquoise and venus, rather than the traditional CFP and YFP. This improved the sensitivity of the sensor making it a very powerful tool for FRET microscopy. However, for the purpose of studying microdomains, membrane specific sensors would be more beneficial. For example, Surdo and colleagues have developed FRET sensors, CUTie, which can be targeted to specific multiprotein complexes at discrete subcellular compartments to reveal differential kinetics and amplitudes of localised cAMP signal thus allowing the study of further accuracy (Surdo et al., 2017).

Furthermore, PDE families are composed of multiple subtypes which are further divided into isoforms. The study of all these subgroups through FRET is not yet possible, as only a handful of inhibitors exist for PDE families which are not specific for isoforms. Thus, differentiating individual isoform activity would require significant progress in the development of inhibitors. Alternatively, conformation of PDE localisation can be obtained by immunostaining or western blots of fractionated membranes.

One major aspect of PDE study is the cross-talk that occurs between cyclic nucleotides. Understanding how cAMP effects cGMP activity, and vice versa, and also the cross-talk between PDEs themselves is essential for understanding how strength and frequency of cardiac contraction and relaxation is regulated. This study only addressed the PDE families independently, however, the inhibition of one PDE may influence the activity of other PDEs or their isoform within those compartments or even in neighbouring domains. Future studies should take this into consideration in order to develop an in depth understanding of PDE and cyclic nucleotide cross-talk.

Summary

iPSC-CMs possess caveolae compartments and show compartmentation of β_2 ARs to a limited extent. PDE functionality is also evident with variable association towards β_1 AR and β_2 AR cAMP pools. This chapter sets sound foundations of the role caveolae play on β AR-cAMP signalling activity in iPSC-CMs as well as the functional activity of the cAMP-degrading PDEs (2, 3 and 4). These assessments have highlighted some of the differences from more mature cells, and support the immaturity noted in previous literature. This chapter accepts the hypothesis that iPSC-CMs possess limited caveolar microdomains and highlights their importance in compartmentalising and maturing β_2 AR-derived cAMP signalling. The data in this chapter can function as a prototype for examining the role of β AR-cAMP signalling activity during iPSC-CMs maturation. One such method in question is by ageing iPSC-CMs in culture, and will be further examined in the next chapter.

CHAPTER 6: EFFECT OF AGEING iPSC-CMs ON CAVEOLAE & β_2 AR- cAMP COMPARTMENTATION

6.1 INTRODUCTION

The previous chapter sets the groundwork for understanding caveolae structures, their associated β AR-cAMP activity and the role of PDEs in iPSC-CMs. In this chapter, the development of the β AR signalling pathway in iPSC-CMs upon time-dependent maturation will be of focus. This will be used for understanding the effectiveness of this maturation technique as a developmental process of iPSC-CMs.

Over the past years, iPSCs differentiation has enhanced dramatically to produce more functional cardiomyocytes and iPSC-CM are becoming invaluable tools for uncovering disease mechanisms, drug toxicity/efficacy and regenerative therapies. However, as depicted in the previous chapters here and in previous literature studies, they prove to be less mature than adult cardiomyocytes. Extending time in culture of iPSC-CMs has previously been reported as to improve cell aspect ratio, sarcomeric organisation, define A-, Z-, M- and I-bands, increase multinucleation, and upregulate various crucial cardiac gene expression (Kamakura et al., 2013; Laflamme & Murry, 2011; Lundy et al., 2013; Snir et al., 2003). Time-dependent maturation has also shown to that CAV3 and Cavin4 gene and protein expression are also dramatically increased (Jung et al., 2016), which is particularly interesting from the perspective of β AR signalling. In addition, improvements in functional properties such as action potential upstroke velocity and amplitude, Ca^{2+} handling with faster upstroke and decay velocity and several other electrophysiological properties have also been identified (Bosman et al., 2013; Lundy et al., 2013).

Furthermore, previous literature on 3D-cultured iPSC-CMs also displayed improved structural, functional and metabolic maturation (Ronaldson-Bouchard et al., 2018b). Mills et al also indicate that uniaxial stretch applied to hESC-CMs and iPSC-CMs led to increased myofibrillogenesis, sarcomeric banding and increased force production (Mills et al., 2017). Similar improvements in calcium handling have also been reported in earlier literature (Krüger et al., 2008; M. Li et al., 2014).

Of particular importance is the development/maturation of the β AR signalling system. The response of β ARs to catecholamine stimulation by coupling G-proteins to adenylyl cyclase and promoting activation of downstream targets are essential to mediate chronotropic and inotropic activity (Steinberg, 1999). Despite it being a primary mechanism of regulating cardiac function, there is limited work on understanding this pathway in iPSC-CMs.

In the previous chapter, the β AR responses and PDE activity towards receptor subtype-dependent cAMP activity was measured; we see that the β AR signalling system has limited receptor expression and is more dependent on β_2 AR than β_1 AR, unlike what is reported in other studies of adult cardiomyocytes. Furthermore, for the first time, this report identifies real-time cAMP handling in iPSC-CMs and demonstrates limited caveolar compartmentation at an early maturation stage, which may be one of the reasons for functional immaturity of iPSC-CMs. Therefore, improving our understanding of the β AR signalling pathway in iPSC-CMs and developing its maturation strategies is essential for scientific and clinical significance.

This chapter will build on the prior knowledge which suggests that there is a time-dependent transition of β AR-subtype specific activity in iPSCs after cardiac induction, and provide insight into the effectiveness of ageing iPSC-CMs as a maturation technique through studying the development of the β AR pathway and its signalling components.

This chapter aims to understand the effect of time-dependent maturation of iPSC-CMs on β AR signalling and PDE2, 3 and 4 activity. iPSC-CM culture time was prolonged from 30 days in culture to 90 days. Changes in gene expression of the functional components of the β AR pathway were assessed, along with the structural development of caveolae, and their role on compartmentalising β ARs-cAMP pools. Furthermore, the gene expression levels of PDE2, 3, and 4 and their functional activity through iPSC-CMs development was assessed.

6.2 METHODS

6.2.1 Cell culture

The iPSC-CMs used in this chapter were derived from the IMR-90 stem cell line and were dissociated, re-plated and cultured as described above. iPSC-CMs were cultured in plastic-bottom culture dishes (either 6-well or 12-well plates) and maintained in RPMI media. iPSC-CMs were dissociated from the plates when required for experiments and re-plated as required into 7mm MatTek dishes. To age the iPSC-CMs, after re-plating at D15, the cells were kept in culture for the required period of time and were used at D30 $\pm$ 5 days, D60 $\pm$ 5 days and D90 $\pm$ 5 days and are referred to as D30, D60 and D90. Culture media was refreshed every 2-3 days.

6.2.2 Cell transfection for CAV3 overexpression

iPSC-CMs (IMR-90 cell line) were transfected at D30 into with the CAV3-RFP plasmid in a similar way to the pTEV FRET sensor. Refer to section 5.2.5.2.

6.2.3 RT-QPCR

6.2.3.1 RNA extraction

RNA was extracted using the RNeasy Mini kit (Qiagen, CA, USA) from cultured hiPSC-CMs according to the manufacturer's guidelines as described above in section 2.3.1.

6.2.3.2 RT-QPCR protocol

Gene expression profiles were investigated in iPSC-CMs at D30 and D90 of culture. iPSCs were plated and differentiated into cardiomyocytes as outline above. Once cells reached the age of 30 or 90 days,

they were harvested and RNA was extracted as described above. The Qiagen kit was used to perform RT-QPCR as described in section 2.3.2. The primer sequences are as follows:

Target Gene	Forward sequence	Reverse sequence
β_1 AR	GACGCTCACCAACCTCTTCA	CACAGCTCGCAGAAGAAGGA
β_2 AR	TTGCCTCTTCCATCGTGTC	CCACCTGGCTAAGGTTCTGG
CAV3	GCGGAAGGAGGTCTAAAGC	AGAGCGAAGGGCCATTGT
PDE2A	CCTCCTGTGACCTCTCTGACC	TGAACTTGTGGGACACCTTGG
PDE3A	TCACAGGGCCTTAACCTACAC	GGAGCAAGAATTGGTTTGTC
PDE3B	CCTCAGGCAGTTTTATACAATG	TGCTTCTTCATCTCCCTGCTC
PDE4A	GTGGAGAAGTCTCAGGTGGG	TGGAACCTGTCAGGCAGGG
PDE4B	TAGAAGATAACAGGAACTGG	GCAATGTCTATGTCAGTCTC
PDE4C	TGATCTGAGCAACCCCAACAG	GGTGAGGTCTGAGGGACTTCG
PDE4D	GGATAATGGAGGAGTTCTTCC	CGATTGTCCTCCAAAGTGTC
BIN1	GCAGGCTCATCTCGTAGCTC	AAACACCTTCTGGGCTTTGA
cTnT	GGCAGCGGAAGAGGATGCTGAA	GAGGCACCAAGTTGGGCATGAACGA
α -Actinin	TCCCTGGAAAAGAGCTACGA	TGCTGTTGTAGGTGGTCTCTCG
JPH2	GACATCGCGAGAGCTGTG	TTCCTGAAATCTCTGTTTGACG
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA

Table 6. 1 Sequences of primers used for qPCR

6.2.3.3 RT-QPCR analysis

Prior to using the QPCR data, the melting curves were checked for the presence of a single peak through the cycle which rose above the threshold. This indicated that the amplified products were of a single species and off-targets products or contamination wasn't occurring. As these didn't occur, the data was used for analysis. The data was normalised to the housekeeping gene GAPDH. The fold differences were calculated through using the " $2^{-\Delta\Delta Ct}$ " method. Statistical significance was tested through the Students T-test.

6.2.4 Immunofluorescence staining

Cells were prepared for immunostaining by seeding them on glass bottom MatTek dishes. They were cultured for 3-4 days to allow recovery and then prepared for CAV3 staining as described in section 2.5.1.

6.2.5 Transmission electron microscopy

6.2.5.1 TEM protocol

Culture medium was removed from cell culture dishes; cells were rinsed with PBS then fixed in 2.5% glutaraldehyde in cacodylate buffer. Further preparation was conducted by Ivan Diakonov as described in section 2.4.1.

6.2.5.2 TEM analysis

From each TEM sample preparation, an “n” number of cells were processed. From each cell, 5-10 sections/frames were prepared. The total number of caveolae per frame was manually counted and used to calculate an average number of caveolae per cell. This was divided by the size of the frame to give the total number of caveolae per μm . To test for statistical significance, a students T-test was conducted.

6.2.6 FRET

6.2.6.1 iPSC-CMs (IMR) transfection with pTEV FRET sensor

iPSC-CMs were transfected with the cAMP-specific FRET sensor plasmid Turquoise-Epac1-Venus (pTEV) plasmid using Lipofectamine3000 kit. The manufacturer’s guidelines were followed with optimization to incubation times (Fig 6.1). As described above in section 5.2.6.2.

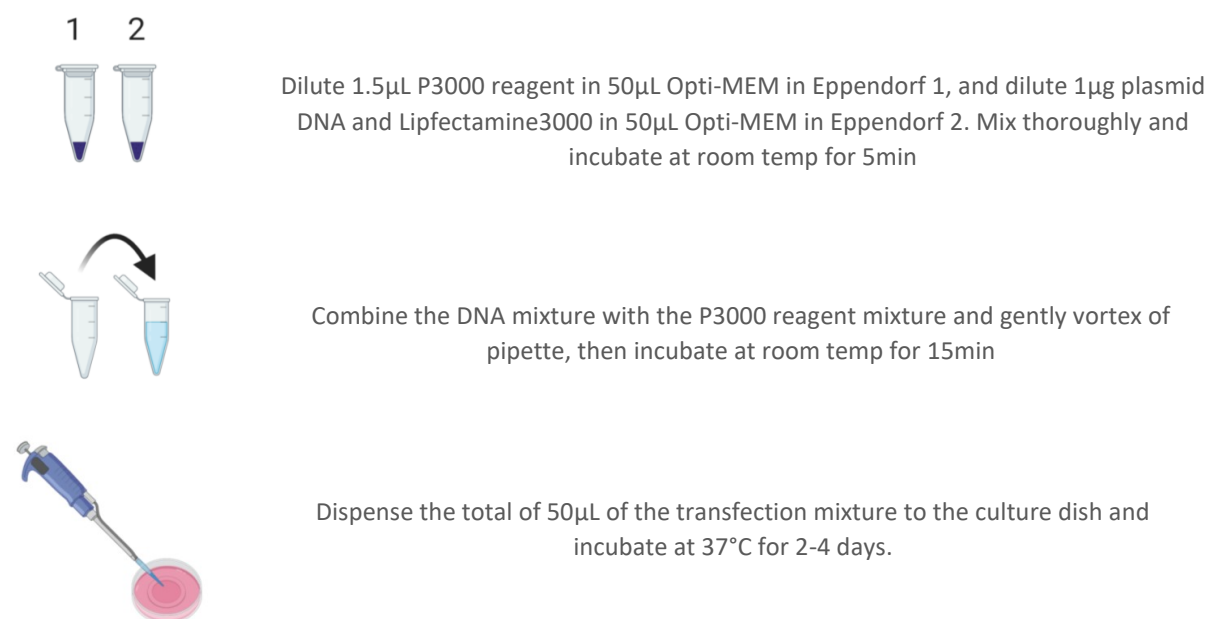


Figure 6. 1 Transfection of iPSC-CMs (IMRs) with the pTEV FRET plasmid

6.2.6.2 Isoprenaline dose-response curves

Dose responses for β AR stimulation with isoprenaline of aged iPSC-CMs (at D90) was conducted to assess the EC_{50} of both receptors using the same protocol as described in Chapter 5. Final concentrations of 0.1nM, 0.3nM, 1nM, 3nM, 10nM, 30nM, and 100nM of ISO were prepared in FRET buffer. CGP20712A (CGP) (Sigma Aldrich, Poole UK) (100nM) was used for blocking β_1 AR and ICI (Tocris, UK) (50nM) was used for blocking β_2 AR prior to ISO stimulation. Upon reaching a plateau in the FRET response, cells were perfused with NKH477 (NKH) (Sigma Aldrich, Poole UK) (final concentration of 10µM) to fully stimulate all AC activity and achieve maximal cAMP release (Fig 6.2).

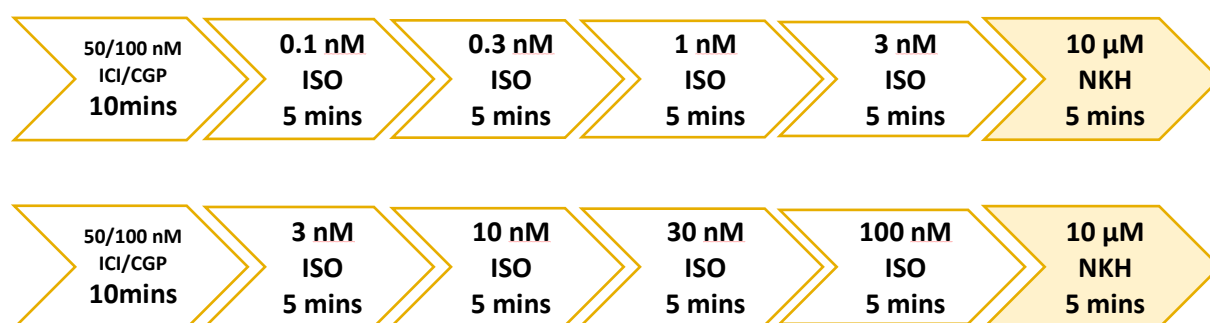


Figure 6. 2 Protocol for dose responses of β_1 AR and β_2 AR to isoprenaline used for FRET

6.2.6.3 Phosphodiesterase inhibition

PDE activity of aged cells (D90) was conducted using the same drug treatment protocol described in Chapter 5. After β_1 AR or β_2 AR stimulation with ISO, PDE2, PDE3 and PDE4 were selective inhibited with 10 μ M EHNA, 10 μ M Cilostamide and 10 μ M Rolipram respectively. Once a plateau was obtained, 100 μ M IBMX was added to the bath for non-selective inhibition of PDEs, followed by 10 μ M NKH (Fig 6.3).

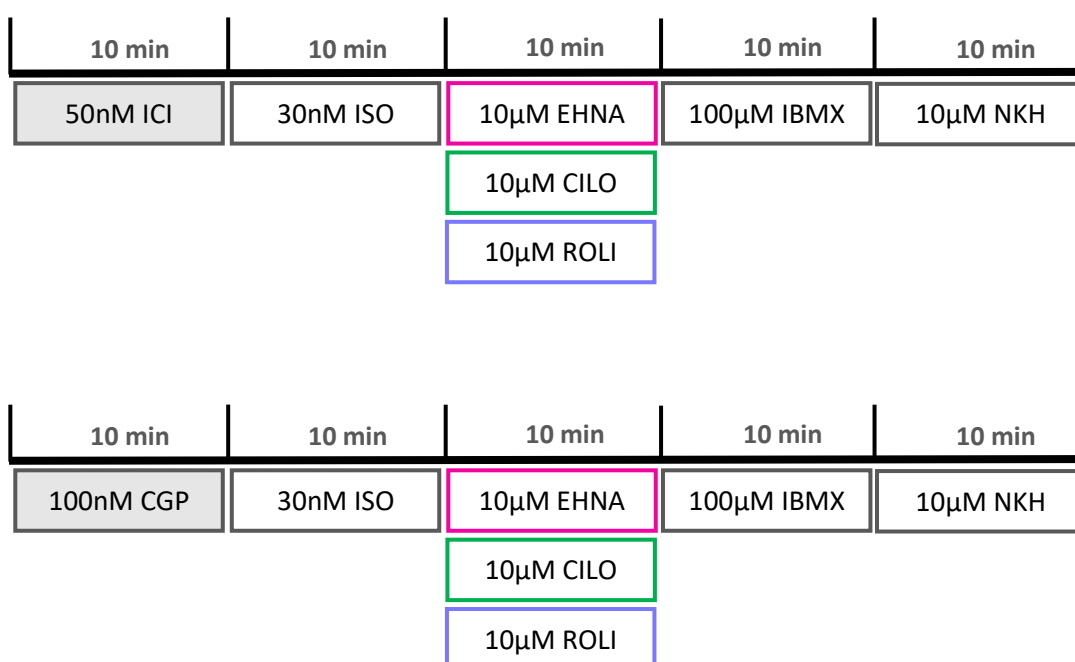
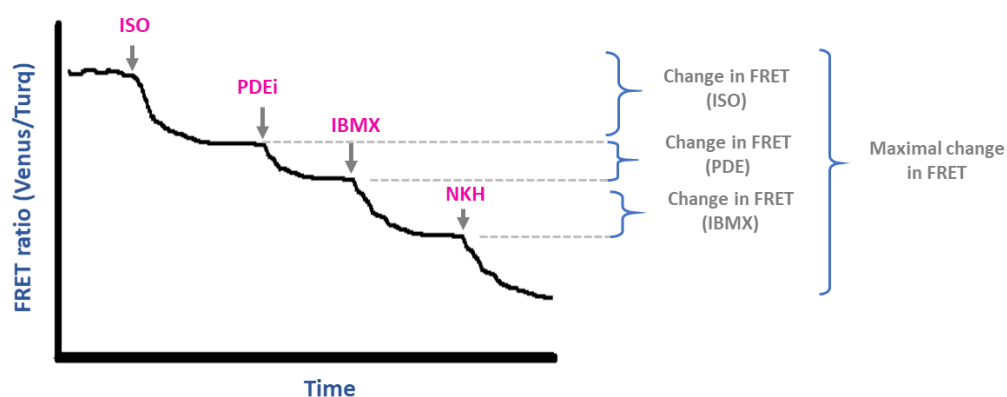


Figure 6. 3 Protocol for investigating PDE2, 3 and 4 activity with FRET microscopy with the respective drug treatments of the stated final concentrations

6.2.6.4 FRET analysis

Analysis of the FRET results were conducted in Microsoft Excel. The ratio between the fluorescence recorded from the YFP channel and the CFP channel was calculated and plotted to obtain a regular YFP/CFP FRET trace. These results are shown as line graphs with the “FRET ratio (Venus/Turquoise)”

plotted. From this the percentage change in the ratio from baseline to the drug treatment in question was calculated. The FRET response from ISO or the PDE inhibitor were normalised to the maximal drop recorded when the cells were treated with NKH. These results are shown as bar graphs plotted as % FRET change Venus/Turquoise normalised to NKH. In addition, the basal tone of the PDE is also reported. This was measured by calculating the change in FRET ratio from β AR stimulation with ISO to PDE drug treatment and is normalised to the maximal cAMP response.



$$\% \text{ FRET change (normalised to NKH)} = \frac{\% \text{ Change in FRET (ISO)}}{\text{Maximal change in FRET}}$$

Maximal change in FRET

$$\% \text{ FRET change (Basal tone)} = \frac{\% \text{ Change in FRET (PDEi)}}{\text{Maximal change in FRET}}$$

Figure 6. 4 Diagram depicting calculations from the FRET traces

6.2.7 Statistical analysis

The statistical tests conducted has been described in each experimental method section. Generally, the statistical analysis used to analyse data obtained for experimental work was Students T-Test, which were performed using GraphPad Prism 5. The statistical significance was set at $p < 0.05$ and data has been reported with $\pm$ SEM (Standard error of the mean).

6.3 RESULTS

6.3.1 Quantification of structural genes expression levels in iPSC-CMs through cardiac differentiation

The gene expression levels of the structural genes, α -actinin, BIN1, JPH2, CAV3 and cTNT were assessed by RT-QPCR in iPSCs (D0) and iPSC-CMs at D30, D60 and D90 of maturation. Expression levels were normalised to the expression levels at iPSCs (D0). There was no clear change in expression levels as iPSC-CMs were aged, except for the expression of cTnT which was significantly increased from D0 to D30 and had the highest expression level observed after iPSCs differentiation ($p < 0.05$). In addition, a small but gradual increase was detected in CAV3 expression levels from D30 to D90, although not statistically significant. A slight reduction in BIN1 was noted, and no change in JPH2 and α -actinin expression was detected (Fig 6.5).

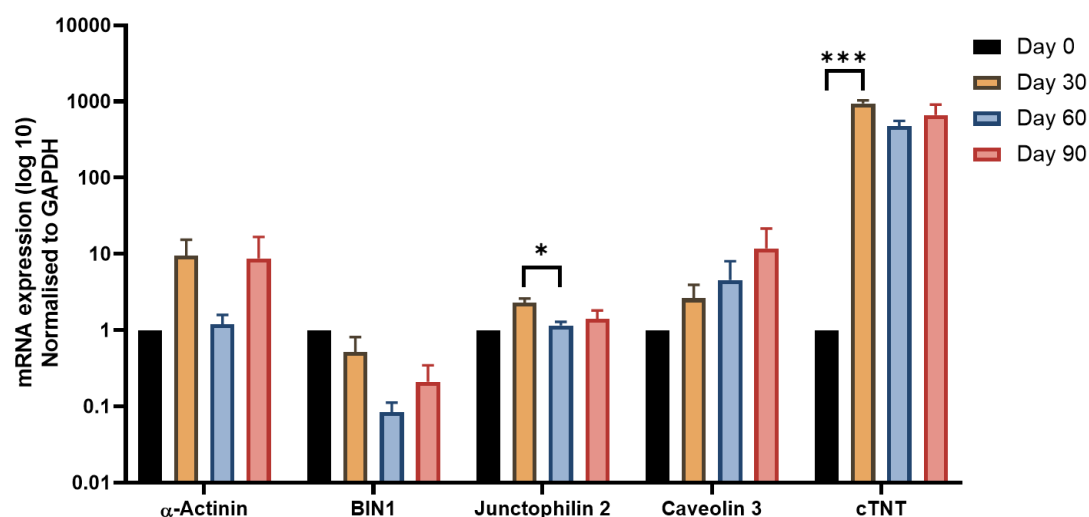


Figure 6. 5 Gene expression analysis of respective genes throughout iPSC maturation

RT-qPCR was used to detect gene expression of the major structural genes for cardiomyocyte structural development. There was no significant difference in gene expression in response to increasing culture time from 30 and 90 days ($n=4$). All results are normalized to GAPDH. (Repeated measures ANOVA, Tukey's post hoc $p < 0.05$). Data presented as mean $\pm$ SEM

6.3.2 Investigating the influence of ageing iPSC-CMs on caveolin-3

Although there was no change in the structural genes assessed previously, the slight increase in CAV3 was investigated further, particularly as it is a crucial player in caveolae structures. The effect of ageing iPSC-CMs from D30 to D90 on CAV3 density and distribution was assessed by immunostaining and imaging for CAV3. M β CD was used to remove membrane caveolae to see if its treatment influences CAV3 density or distribution. As previously described, ageing iPSC-CMs to D90 led to an increase in CAV3 gene expression, although statistical significance was not obtained (Fig 6.6a). Immunofluorescence staining images were used to calculate CAV3 protein density. There was a 45% increase in CAV3 density after ageing cells. There was no change from control in protein density detected when cells were treated with M β CD at D30 or D90 (Fig 6.6b, c).

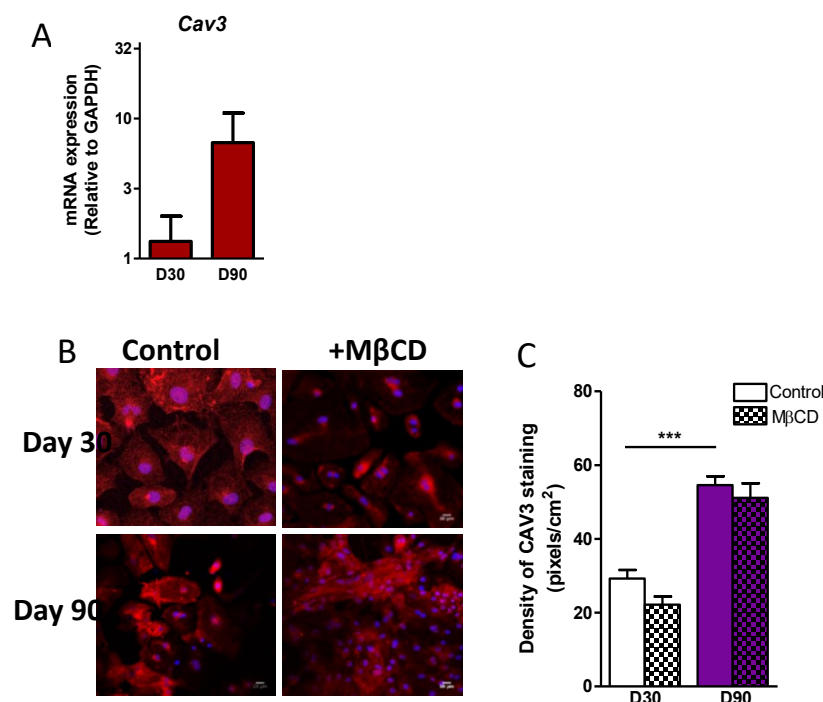


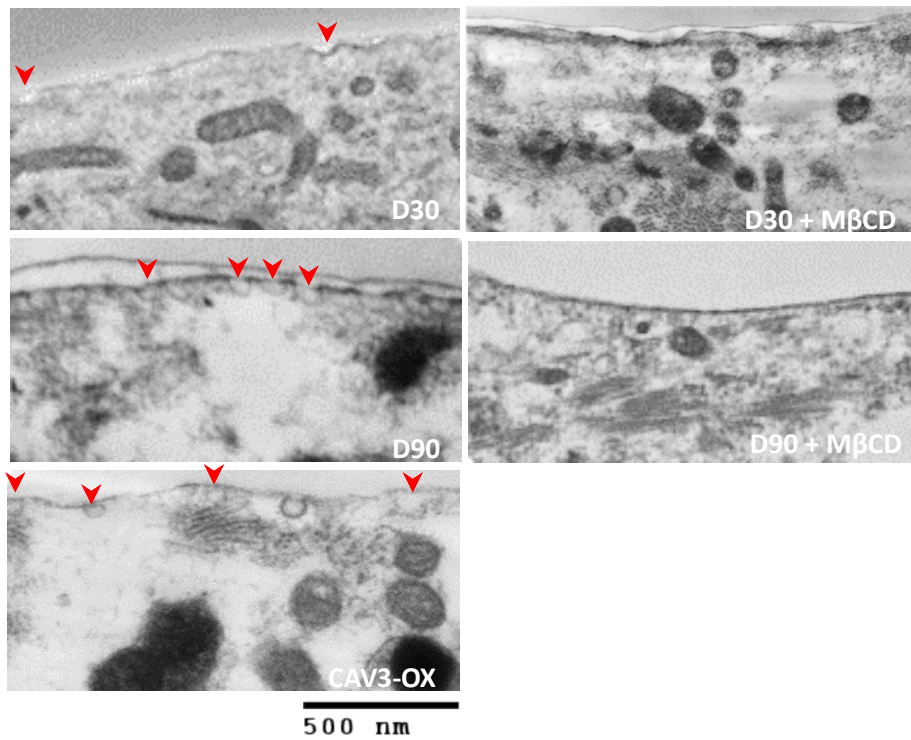
Figure 6. 6 CAV3 expression and protein density of age-dependent maturation of iPSC-CMs

A) Gene expression of CAV3 in D30 and D90 iPSC-CMs ($n=6$). **B)** Immunofluorescence staining for CAV3 (red) and DAPI (blue) at D30 and D90 iPSC-CMs. **C)** Quantification of CAV3 protein density at D30 ($n=19$) to D90 ($n=13$). Prolonging culture period of iPSC-CMs to 90 days after differentiation increases CAV3 gene expression and protein density. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, *** $p<0.001$

6.3.3 Assessing the influence of ageing iPSC-CMs on caveolar compartments

As ageing iPSC-CMs significantly increases CAV3 protein density, its effect on membrane caveolae structures was assessed. The density of caveolae compartments were investigated through transmission electron microscopy (TEM). Control and M β CD-treated iPSC-CMs at D30 and D90 were processed for TEM. Analysis showed that at D30 under control conditions, iPSC-CMs possess 0.2 caveolae per μm . After ageing to D90, this increases to 0.6 caveolae per μm . This is a 3-fold increase in caveolae number after ageing. When cells were treated with M β CD and then assessed for membrane caveolae content, there was a significant decrease in caveolae number in both the control D30 and the aged D90 cells; dropping to 0.1 and 0.08 caveolae per μm respectively ($p < 0.05$). In comparison to when cells are transfected with CAV3 for overexpression of the protein, a significant increase from control in membrane caveolae is observed ($p < 0.05$), although not as extensively as D90 cells (Fig 6.7).

A



B

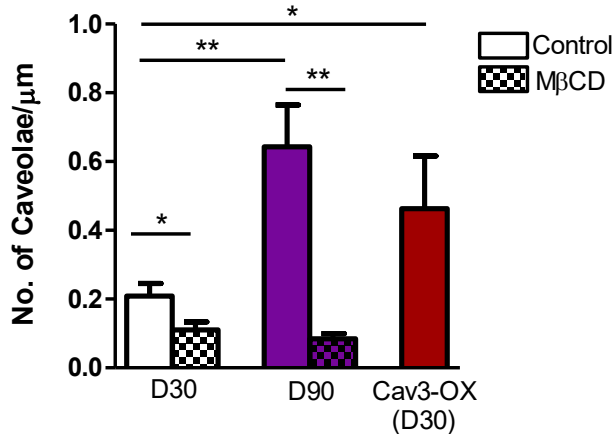


Figure 6. 7 Effect of age-dependent maturation on iPSC-CMs membrane caveolae number

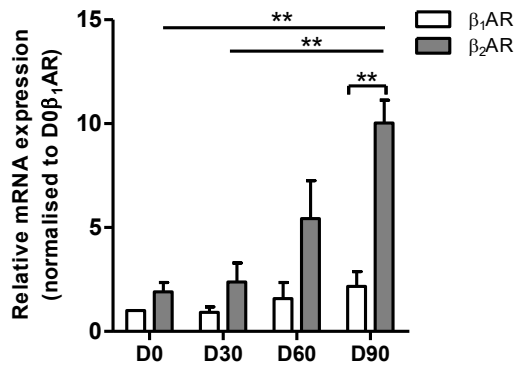
A) TEM images showing surface caveolae (indicated with arrows) in control $\pm$ M β CD treatment, aged iPSC-CMs to D90 $\pm$ M β CD and in CAV3-OX (D30) iPSC-CMs. Scale bar represents 0.5 μ m. B) Quantification of caveolae per μ m of cell membrane in D30 control (n= 45/2), D30 M β CD-treated (n=37/2), D90 control (n= 10/2), D90 M β CD-treated (n=13/2) and CAV3-OX (n=21/1) iPSC-CMs. Ageing the cells in culture significantly increases the number of membrane caveolae complexes to levels similar to overexpressing CAV3. The number of caveolar compartments decrease significantly when iPSC-CMs are treated with M β CD. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. *p<0.05, ***p<0.001. n=cells/batches

6.3.4 Investigating the effect of ageing iPSC-CMs on the genetic and functional activity of β_1 ARs and β_2 ARs

As ageing iPSC-CMs to D90 has shown to increase membrane caveolae, the influence this has on the functional activity of β AR signalling and cAMP activity was investigated. To begin with, the expression levels of β_1 AR and β_2 AR were quantified through RT-qPCR of iPSCs (D0) and of iPSC-CMs at D30, D60 and D90 of maturation. Interestingly only a 1.4-fold increase in β_1 AR expression was observed from D30 to D90 iPSC-CMs and no statistical significance was obtained; interestingly, there was a dramatic increase of 3.2-fold in β_2 AR expression was obtained as cells were aged to D90. At D30, β_2 AR expression was 1.7-fold higher than β_1 AR, and at D90 β_2 AR expression was 3.5-fold greater than β_1 AR (Fig 6.8a).

To assess the influence of increased CAV3 expression and increased caveolae microdomains as iPSC-CMs are aged, cAMP activity was assessed in D30 and D90 in response to β_1 AR or β_2 AR stimulation with FRET microscopy. β ARs were stimulated with isoprenaline in the presence of ICI or CGP respectively. Isoprenaline concentration ranging from 0.1-100nM were tested from which a dose response curve was produced. When β_1 AR was stimulated with isoprenaline, an EC_{50} of is 4.8nM and 14.4nM was obtained in D30 and D90 cells respectively; a statistically significant difference was obtained ($p < 0.05$). On the other hand, in the dose response curve upon β_2 AR stimulation, an EC_{50} of 2.4nM and 7.7nM in D30 and D90 respectively was calculated; no statistical difference was observed. There was no change in the maximal response of the β_1 AR response from D30 to D90, however the β_2 AR response was significantly increased from D30 to D90 ($p < 0.05$) (Fig 6.8b).

A



B

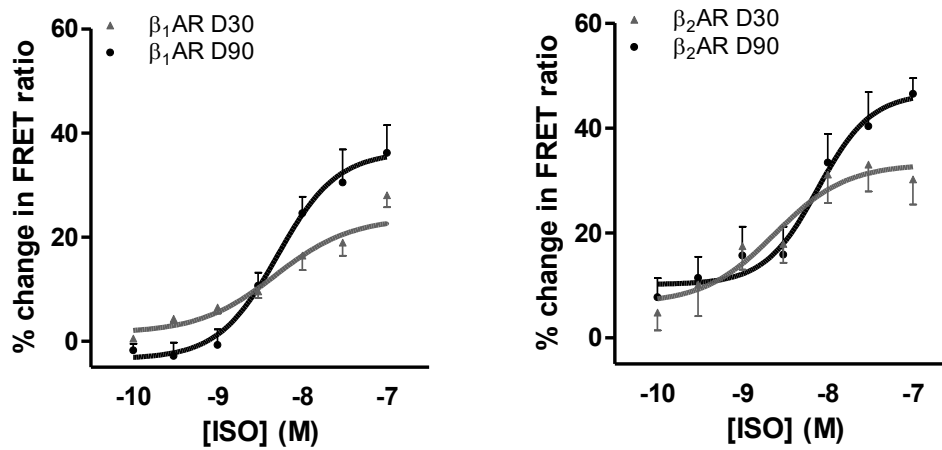


Figure 6. 8 Stimulation of β AR with isoprenaline induces dose-dependent cAMP release

A) Differential β_1 AR and β_2 AR gene expression in iPSCs (D0) and iPSC-CMs after 30, 60 and 90 days of differentiation. N=3 B) Concentration response curves for β_1 AR and β_2 AR to Isoprenaline ranging from 0.1nM to 100nM at D30 and D90 of iPSC differentiation. EC50 for β_1 AR at D30 vs D90 is 4.8nM and 14.4nM respectively ($F(n,d)=30.55$ (4,227), $p<0.0001$), and for β_2 AR at D30 vs D90 is 2.4nM and 7.7nM respectively ($f(n,d)=1.55$ (4,66), $p=0.2$) Data shown as mean $\pm$ SEM; * $P<0.05$, ** $P<0.01$ between marked groups (1-way ANOVA with Tukey's post hoc test).

6.3.5 The influence of ageing iPSC-CMs on the functional activity of β_1 AR-cAMP

Next, FRET experiments were conducted to investigate how changes in membrane caveolae as iPSC-CMs were aged may influence β_1 AR-dependent cAMP signalling. β_1 ARs were stimulated with isoprenaline in the presence of the β_2 AR-blocker ICI, PDEs were subsequently inhibited by IBMX, and then treated with NKH for maximal cAMP output. Results were normalised to the NKH response. Upon β_1 AR stimulation of D30 cells, a $23.7 \pm 2\%$ cAMP response was obtained. The addition of IBMX significantly elevated the level of cytosolic cAMP to around 80% of the total cAMP potential of the cell indicated significant PDE activity ($p < 0.05$). A similar response was obtained from D60 and D90 cells; therefore, no difference from D30 was observed as the iPSC-CMs were aged. The cAMP response detected after IBMX treatment was relatively consistent at all three ages of cells (Fig 6.9).

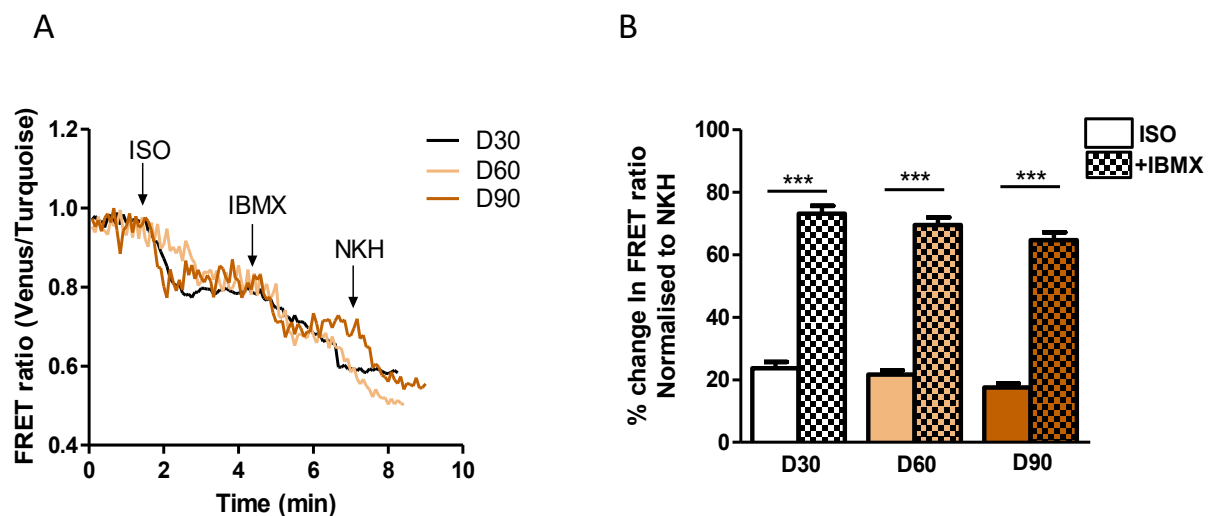


Figure 6. 9 β_1 AR-dependent cAMP output throughout iPSC-CM maturation

A) Representative FRET traces of D30, D60 and D90 iPSC-CMs upon β_1 AR stimulation with ISO in the presence of β_2 AR blocker ICI, PDE inhibition with IBMX and subsequent activation of AC with NHK477. B) Percentage change in FRET ratio normalised to maximal cAMP released by NKH stimulation D30 ($n = 97/11$), D60 ($n = 158/11$) and D90 ($n = 121/8$) for β_1 AR responses. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, *** $p < 0.001$. $n = \text{cells/batches}$

6.3.6 The influence of ageing iPSC-CMs on the functional activity of β_2 AR-cAMP

Similar to the previous experiment, how ageing iPSC-CMs changes membrane caveolae number may influence β_2 AR-dependent cAMP signalling was investigated. β_2 ARs were stimulated with isoprenaline in the presence of the β_1 AR-blocker CGP, PDEs were subsequently inhibited by IBMX, and then treated with NKH for maximal cAMP output. Results were normalised to the NKH response. Upon β_2 AR stimulation with isoprenaline of D30 cells, a $47.7 \pm 4.5\%$ cAMP response was recorded. In D60 cells, a decrease in cAMP was recorded at $35.2 \pm 5.9\%$ output of total cAMP. Interestingly, by D90, only an $11.5 \pm 2.2\%$ change in FRET ratio was recorded in response to β_2 AR stimulation; this was a 75% lower than D30 cells. This was a significant drop from D30 and D90 iPSC-CMs ($p < 0.05$). PDE inhibition with IBMX resulted in an 80% change in FRET ratio, this was significantly increased from the cAMP upon β_2 AR stimulation and was consistent at all 3 ages ($p < 0.05$) (Fig 6.10). This indicates that background PDE's are also functionally active against β_2 AR-cAMP pools.

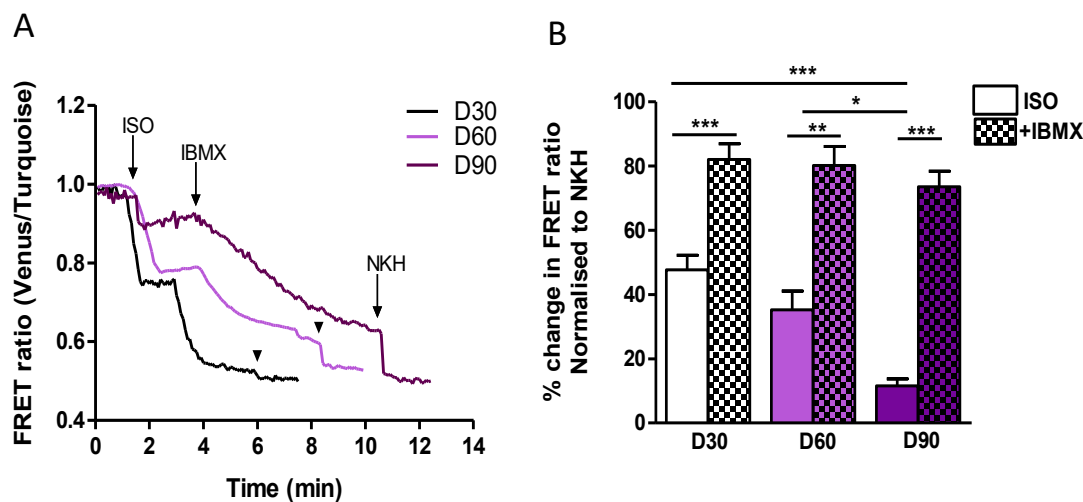


Figure 6. 10 β_2 AR-dependent cAMP output throughout iPSC-CM maturation

A) Representative FRET traces of D30, D60 and D90 iPSC-CMs upon β_2 AR stimulation with ISO in the presence of β_1 AR blocker, PDE inhibition with IBMX and activation of AC with NHK477. B) Percentage change in FRET ratio normalised to maximal cAMP released by NKH stimulation at D30 ($n = 38/4$), D60 ($n = 15/3$) and D90 ($n = 90/5$) for β_2 AR responses. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, *** $p < 0.001$. $n = \text{cells/batches}$

6.3.7 Investigating caveolar compartmentation of β_1 AR and β_2 AR- dependent cAMP activity in aged iPSC-CMs

To understand more specifically the role that caveolae play in β_1 AR and β_2 AR signalling in aged iPSC-CMs, M β CD was used to remove caveolae and then changes in cAMP output was assessed. Results were normalised to NKH treatment to understand cAMP output in relation to maximal cAMP output by AC activation. There was a significant increase detected in cAMP activity in relation to maximal output from both β_1 AR (from $17.6 \pm 1.3\%$ to $29.0 \pm 2.8\%$ FRET change) and β_2 AR (from $11.5 \pm 2.2\%$ to $64.2 \pm 3.9\%$ FRET change) after caveolae depletion ($p < 0.05$); although the increase in β_2 AR is more dramatically augmented (Fig 6.11).

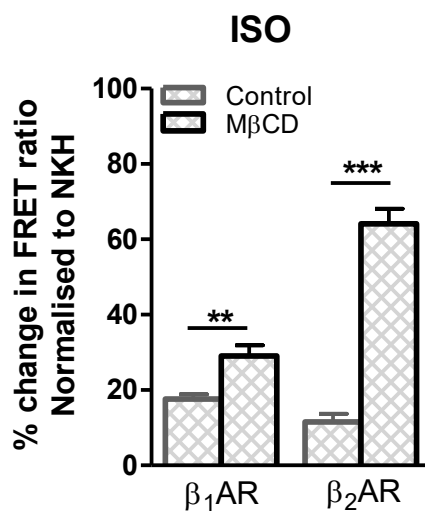


Figure 6. 11 β_1 AR and β_2 AR activity in caveolar domains of aged iPSC-CMs

The association of β AR-cAMP activity with caveolae was assessed in iPSC-CMs at D90 with isoprenaline in the presence of ICI or CGP for β_1 AR and β_2 AR respectively. The percentage change in FRET ratio was normalised the maximal cAMP produced upon AC activation with NKH. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

6.3.8 Effect of ageing iPSC-CMs on PDE2, PDE3 and PDE4 gene expression

Ageing iPSC-CMs significantly increases CAV3 protein and membrane caveolae number, and this correlates well with the decrease in β_2 AR signalling. This indicates that age-dependent maturation effectively increases cAMP compartmentation. This study continues by investigating whether changes in PDE activity are also affected by prolonged iPSC-CMs culture.

From previous experiments, it was interesting to note that despite the changes in cAMP handling after ageing iPSC-CMs, there was no detectable change to overall PDE responses by IBMX treatment. However, the relative contribution of individual PDE families was not depicted. To investigate further, PDE2, 3, and 4 gene expression profiles were assessed by RT-qPCR of iPSCs and iPSC-CMs at D30, D60 and D90 of maturation. Results were normalised to iPSCs (D0). PDE2A had an expression level of 5.7 at D30, which lowered to 3.2 by D90; although these results were not significantly different. PDE3A had an expression level of 3.1 at D30 and 4.8 at D90. PDE3B also had similar expression levels at D30 and D90; which seemed to be lower than the expression level of PDE3A. PDE3B seemed to be the least expressed subtype in iPSC-CMs at D30. Of the PDE4 family, PDE4D was the most highly expressed, significantly higher than the other PDE subtypes ($p < 0.05$). Overall, when investigating the effect of ageing iPSC-CMs on PDE expression profiles, no significant changes from D30 to D90 were observed.

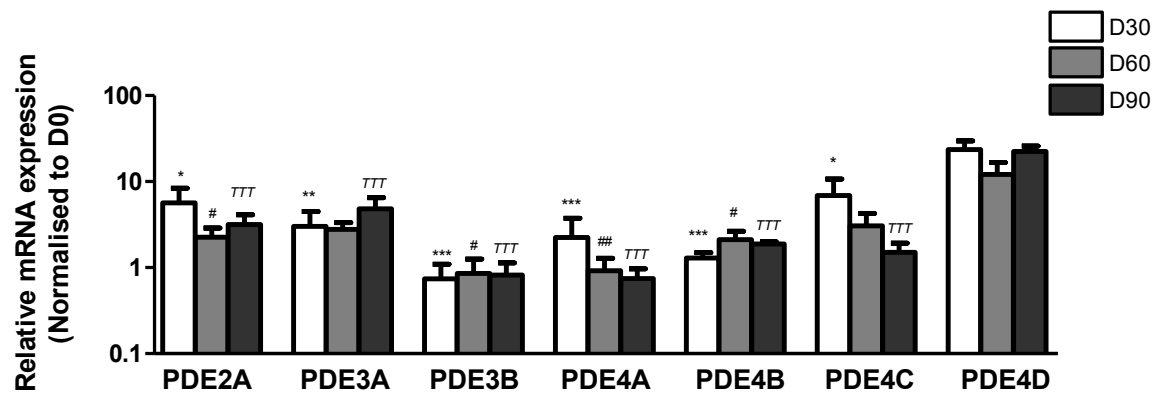


Figure 6. 12 Gene expression profiles of PDE 2, 3, 4 isoforms at D30, D60 and D90 in culture

RT-QPCR was used to detect gene expression of three major PDE 2, 3, 4 families. There was no significant difference in gene expression of these PDEs from 30 to 90 days. A significant difference was observed from PDE4D with all other PDE subtypes at D30 (*), D60 (#) and D90 (T) respectively. One-way ANOVA, Bonferroni's post hoc ($p < 0.05$). Data presented as mean $\pm$ SEM.

6.3.9 β_1 AR and β_2 AR-cAMP dependent PDE2 activity in aged iPSC-CMs

Firstly, the influence of caveolar compartmentation on PDE2 activity upon β_1 AR and β_2 AR cAMP pools in aged cells (at D90 of differentiation) was assessed through FRET microscopy. M β CD was used for removing caveolae from the cell membrane. β ARs were stimulated with isoprenaline in the presence of either ICI or CGP to achieve a β_1 AR- or β_2 AR-cAMP response respectively. Cells were treated with EHNA for inhibition of PDE2. Subsequently, all PDEs were inhibited by treatment with IBMX, and finally ACs were activated by NKH for maximal cAMP output. Change in FRET ratio was normalised to NKH response to obtain a response relative to the total cellular cAMP activity. This experiment was conducted in control and M β CD-treated conditions.

In control cells, treatment with EHNA increased β_1 AR-cAMP output from $17.6 \pm 1.3\%$ to $25.2 \pm 3.6\%$; although this was not statistically significant. Upon removal of caveolar compartments, β_1 AR-cAMP output became significantly increased to $29.0 \pm 2.8\%$ and $43.4 \pm 5.6\%$ upon PDE2 inhibition ($p < 0.05$). Similarly, PDE2 activity on β_2 AR-cAMP pool was significantly enhanced after caveolae disruption ($p < 0.05$). cAMP output was increased from $11.6 \pm 2.2\%$ in control to $64.2 \pm 4.0\%$ in M β CD-treated cells upon β_2 AR stimulation with ISO, and from 38% in control to 66% in M β CD-treated cells after EHNA.

The role of PDE2 on degrading β_1 AR- and β_2 AR-dependent cAMP was assessed by calculating the change in FRET ratio due to the addition of EHNA alone, this is reported as basal tone. Interestingly, PDE2 showed a greater role in β_1 AR-dependent cAMP degradation than β_2 AR-dependent cAMP. Upon treatment with M β CD for caveolae removal, there was a difference detected in the tone of PDE2 on β_2 AR-cAMP. This was greater effect of PDE2 inhibition detected after M β CD treatment; although not significant, possibly due to the large error bars (6.13d).

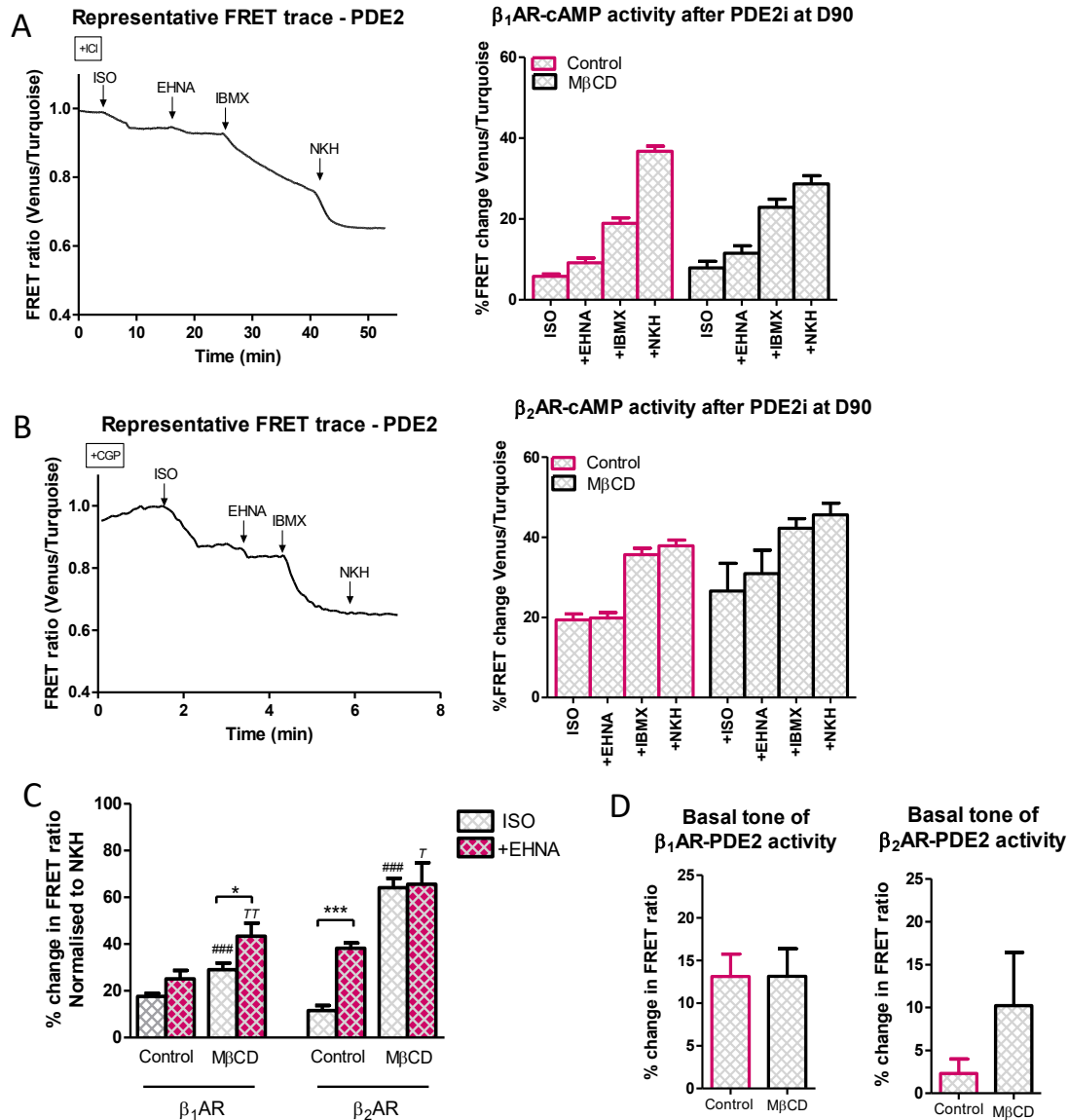


Figure 6.13 β_1 AR and β_2 AR dependent PDE2 activity in D90 iPSC-CMs

The association of β_1 AR and β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D90 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE2 with EHNA. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β_1 AR stimulation and quantified percentage FRET change in control ($n=48/3$) and MβCD ($n=9/2$). B) Representative FRET trace upon β_2 AR stimulation and quantified percentage FRET change in control ($n=55/7$) and MβCD ($n=8/2$). C) Comparison of β_1 AR and β_2 AR response to PDE2 inhibition in control and MβCD treated cells where the response is normalised to the effect of NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and MβCD treated cells. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

6.3.10 β_1 AR and β_2 AR-cAMP dependent PDE3 activity in aged iPSC-CMs

Next, the influence of caveolar compartmentation on PDE3 activity on both β_1 AR- and β_2 AR-cAMP pools in aged cells was also assessed in a similar way to the previous experiment. β_1 AR- or β_2 AR-cAMP were stimulated with isoprenaline before the inhibition of PDE3 with cilostamide. IBMX was used for the inhibition of all intracellular PDEs and NKH was used for AC activation. This experiment was conducted in control and M β CD-treated conditions in iPSC-CMs at D90 of differentiation.

Interestingly, in response to cilostimide, there was a decrease in cAMP output from that of β_1 AR stimulation with isoprenaline from $17.6 \pm 1.3\%$ to $2.8 \pm 2.8\%$. Upon caveolae disruption with M β CD, a similar response was obtained, the FRET ratio decreased from $29.0 \pm 2.8\%$ to $11.2 \pm 6.3\%$. The decrease in FRET ratio indicates that inhibition of PDE3 leads to a reduction in cellular cAMP. This decrease in response was not detected within β_2 AR-cAMP pools. PDE3 inhibition after β_2 AR activation led to a significant increase in cAMP output, from $11.54 \pm 2.2\%$ to $60.7 \pm 5.7\%$ in control cells ($p < 0.05$). As the disruption of caveolae microdomains had significantly augmented the cAMP response upon β_2 AR stimulation ($p < 0.05$), only a slight increase was detected after PDE3 inhibition (Fig 6.14a, b, c).

The role of PDE3 on degrading β_1 AR- and β_2 AR-dependent cAMP was assessed by calculating the change in FRET ratio due to the addition of cilostamide alone, this is reported as basal tone. Interestingly, PDE3 influenced cAMP in an opposing manner from PDE2 activity. Cilostamide addition lead to an increase in FRET ratio, indicating a decrease in cAMP concentrations (Fig 6.14d).

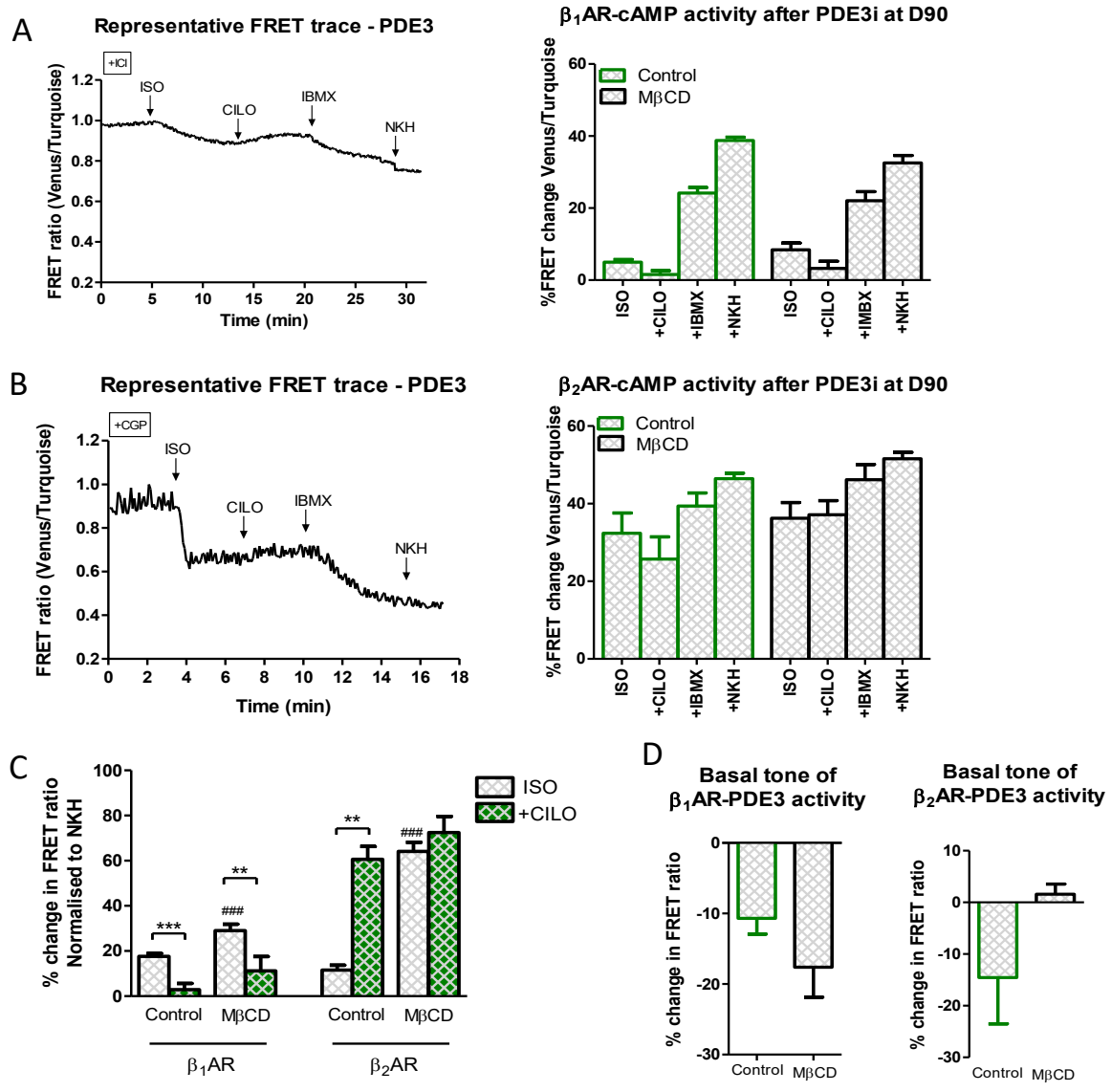


Figure 6. 14 β_1 AR and β_2 AR-dependent PDE3 activity in D90 iPSC-CMs

The association of β_1 AR and β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D90 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE3 with Cilostamide. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β_1 AR stimulation and quantified percentage FRET change in control ($n=51/2$) and MβCD ($n=17/2$). B) Representative FRET trace upon β_2 AR stimulation and quantified percentage FRET change in control ($n=8/3$) and MβCD ($n=9/3$). C) Comparison of β_1 AR and β_2 AR response to PDE3 inhibition in control and MβCD treated cells where the response is normalised to the effect of NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and MβCD treated cells. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

6.3.11 β_1 AR and β_2 AR-cAMP dependent PDE4 activity in aged iPSC-CMs

Lastly, the influence of caveolar compartmentation on PDE4 activity on both β_1 AR- and β_2 AR-cAMP degradation in aged cells was also assessed as previously described. PDE4 was inhibited with rolipram. This experiment was conducted in control and M β CD-treated conditions in iPSC-CMs at D90 of differentiation.

In control conditions, β_1 AR activation with PDE4 inhibition resulted in an $73.0 \pm 4.8\%$ cAMP output of total PDE activity, and a small yet non-significant increase in FRET from $73.1 \pm 4.7\%$ to $82.3 \pm 2.6\%$ was detected when caveolae were removed. PDE4 activity on β_2 AR-cAMP resulted in maximal release of cAMP (98% FRET shift) in control conditions, such that the effect of caveolae removal was undetectable (Fig 6.15 a, b, c).

The role of PDE4 on degrading β_1 AR- and β_2 AR-dependent cAMP was assessed by calculating the change in FRET ratio after the addition of rolipram alone, this is reported as basal tone. PDE4 showed a greater role in β_1 AR-dependent cAMP degradation than β_2 AR-dependent cAMP. PDE4 was responsible for $60 \pm 4.7\%$ of all PDE activity, and the removal of caveolae had no significant influence on this. A smaller effect of PDE4 was detected on β_2 AR-dependent cAMP, and caveolae removal increased PDE4-dependent cAMP degradation from $20 \pm 5.1\%$ to $40 \pm 5.6\%$ total activity (Fig 6.15d).

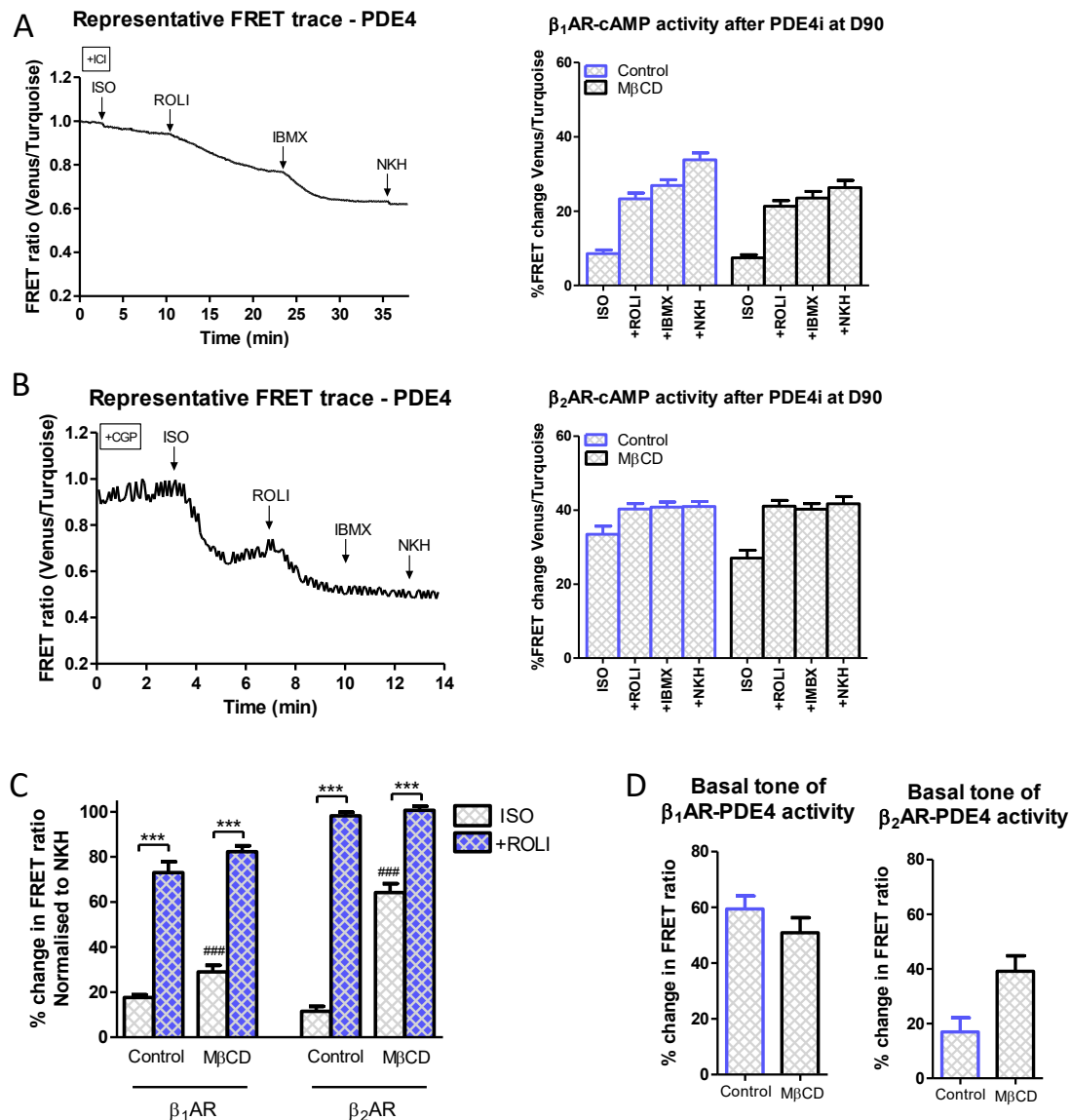


Figure 6. 15 β_1 AR and β_2 AR-dependent PDE4 activity in D90 iPSC-CMs

The association of β_1 AR and β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D90 with isoprenaline in the presence of ICI or CGP respectively, with subsequent inhibition of PDE4 with Rolipram. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. A) Representative FRET trace upon β_1 AR stimulation and quantified percentage FRET change in control ($n=36/3$) and M β CD ($n=23/2$). B) Representative FRET trace upon β_2 AR stimulation and quantified percentage FRET change in control ($n=33/3$) and M β CD ($n=40/3$). C) Comparison of β_1 AR and β_2 AR response to PDE4 inhibition in control and M β CD treated cells where the response is normalised to the effect of NKH. D) The percentage change in FRET ratio resented to show the basal tone of the PDE effect in control and M β CD treated cells. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. n =cells/batches

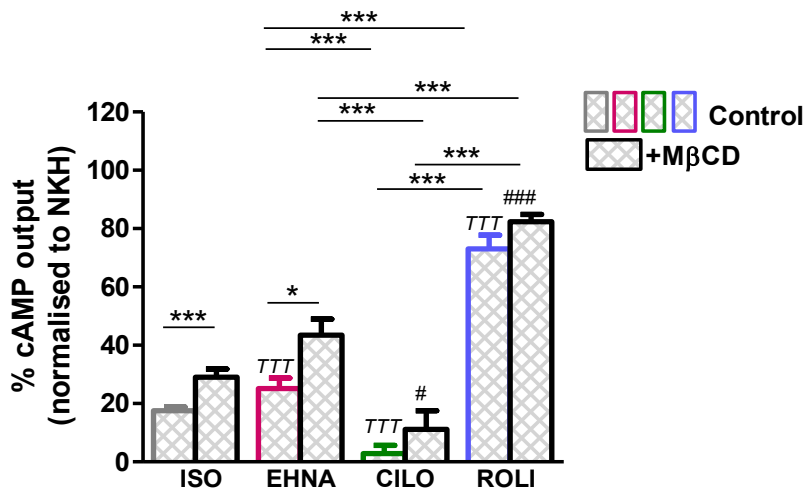
6.3.12 Comparing β AR-cAMP dependent PDE2, PDE3 and PDE4 activity in aged iPSC-CMs

A comparison between the three PDEs of aged iPSC-CMs was made to understand the role of each PDE and its association with caveolar microdomains. In the absence of PDE inhibitors, 18% of maximal cAMP is maintained in the cytosol after there β_1 AR stimulation with isoprenaline alone. Inhibiting PDE2 activity slightly, yet significantly, raised cytosolic cAMP level to 25% cellular cAMP output ($p<0.05$). PDE4 has the greatest response leading to 73% of total cAMP output. Interestingly, PDE3 inhibition showed to have a negative influence on cAMP output and actually reduced cellular cAMP to less than 3%. After the removal of caveolae using M β CD, β_1 AR stimulation led to a 30% cAMP output, significantly raised from control ($p<0.05$). The removal of caveolae only significantly influence PDE2 activity ($p<0.05$), almost doubling cAMP output after its inhibition. Little effect was observed on PDE3 and PDE4 inhibition in comparison to control, -M β CD cells (Fig 6.16a).

The PDEs involved with β_2 AR-dependent cAMP degradation was also investigated. β_2 AR stimulation in the absence of PDE inhibitors led to a 12% cytosolic cAMP level. Inhibition of PDE2 led to a 3-fold increase in cAMP accumulation. Inhibition of PDE3 had a greater effect, leading to a 6-fold increase in cAMP. Inhibition of PDE4 had the greatest influence on cAMP, leading to an almost maximal cAMP output. The removal of caveolae releases 64% of total cAMP when PDEs are still in play. There is no further change seen when PDE2 or PDE3 were inhibited. However, PDE4 inhibition led to maximal cAMP output being achieved (Fig 6.16b).

A

β_1 AR-dependent PDE activity at D90



B

β_2 AR-dependent PDE activity at D90

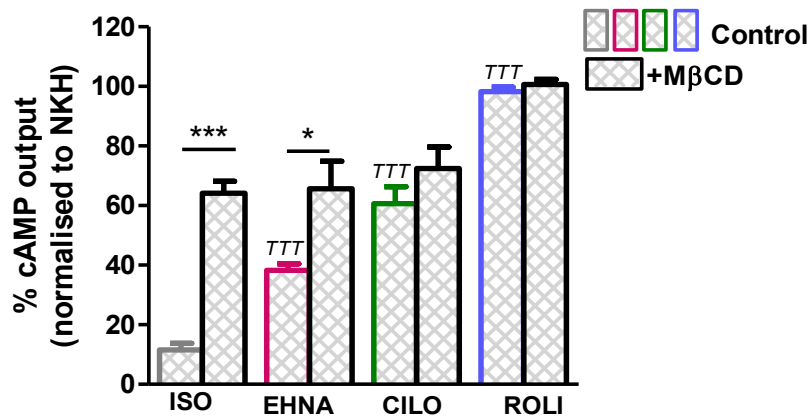


Figure 6. 16 β_1 AR- PDE2, 3 and 4 activity in in control and MβCD-treated D90 iPSC-CMs

A) The association of β_1 AR and B) β_2 AR-cAMP degrading PDE activity with caveolae was assessed in iPSC-CMs at D90 with isoprenaline in the presence of ICI, with subsequent inhibition of PDE2, 3 or 4 with EHNA, Cilostamide or Rolipram. The percentage change in FRET ratio was normalised maximal cAMP release upon AC activation by NKH. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

6.3.13 PDE2, PDE3 and PDE4 activity on β_1 AR- and β_2 AR-dependent cAMP pools in D30 and D90 iPSC-CMs

Although there were no changes detected in PDE gene expression profiles as iPSC-CMs were aged from D30 to D90, it was important to depict any potential underlying functional activity. After either β_1 AR or β_2 AR stimulation with isoprenaline in D30 and D90 cells, PDE2, 3 and 4 activity was assessed through using selective inhibitors, EHNA, Cilostamide (CILO) and Rolipram (ROLI) respectively. Stimulation of β_1 AR with isoprenaline in the absence of PDE inhibitions led to a 23% cAMP output in D30 cells compared to 17% in D90 cells. No statistically significant difference in PDE2, PDE3 or PDE4 activity, and therefore, cAMP output, was detected by ageing iPSC-CMs from D30 to D90. In summary, in comparison to β_1 AR-cAMP from ISO stimulation, PDE2 inhibition did not affect cAMP output. Inhibition of PDE3 lowered cellular cAMP to only about a 2% of total cytosolic cAMP; and inhibition of PDE4 had the largest increase in cAMP leading to around 75% cAMP output (Fig 6.17a).

Investigating the β_2 AR-cAMP dependent PDE activity as aged cells had very interesting responses in comparison to β_1 AR activity. Firstly, upon assessing solely the activity of β_2 AR stimulation alone before and after ageing, a significant decrease in cAMP output from 44% to 11% of total cellular cAMP was detected ($p < 0.05$). At D30, stimulation of β_2 AR with the inhibition of PDE2 lead to a 51% cAMP output. This PDE2 activity was significantly reduced in D90 cells with only a 38% cAMP output detected ($p < 0.05$). When investigating the influence of PDE3, a significant increase from 30% to 60% change in FRET was observed after ageing iPSC-CMs ($p < 0.05$). PDE4 activity was also significantly increase in D90 cells from 70% to 99% change in FRET ($p < 0.05$). In summary, as mentioned in the previous chapter, at D30, in comparison to receptor stimulation with ISO in the absence of PDE inhibitors, no change in cAMP was detected after PDE2 inhibition, PDE3 inhibition significantly reduced cytosolic cAMP ($p < 0.05$), and PDE4 inhibition significantly increase cytosolic cAMP ($p < 0.05$). However, at D90, inhibition of PDE2, 3 and 4 resulted in a significant increase cellular cAMP output in comparison to receptor stimulation with isoprenaline alone ($p < 0.05$) (Fig 6.17b).

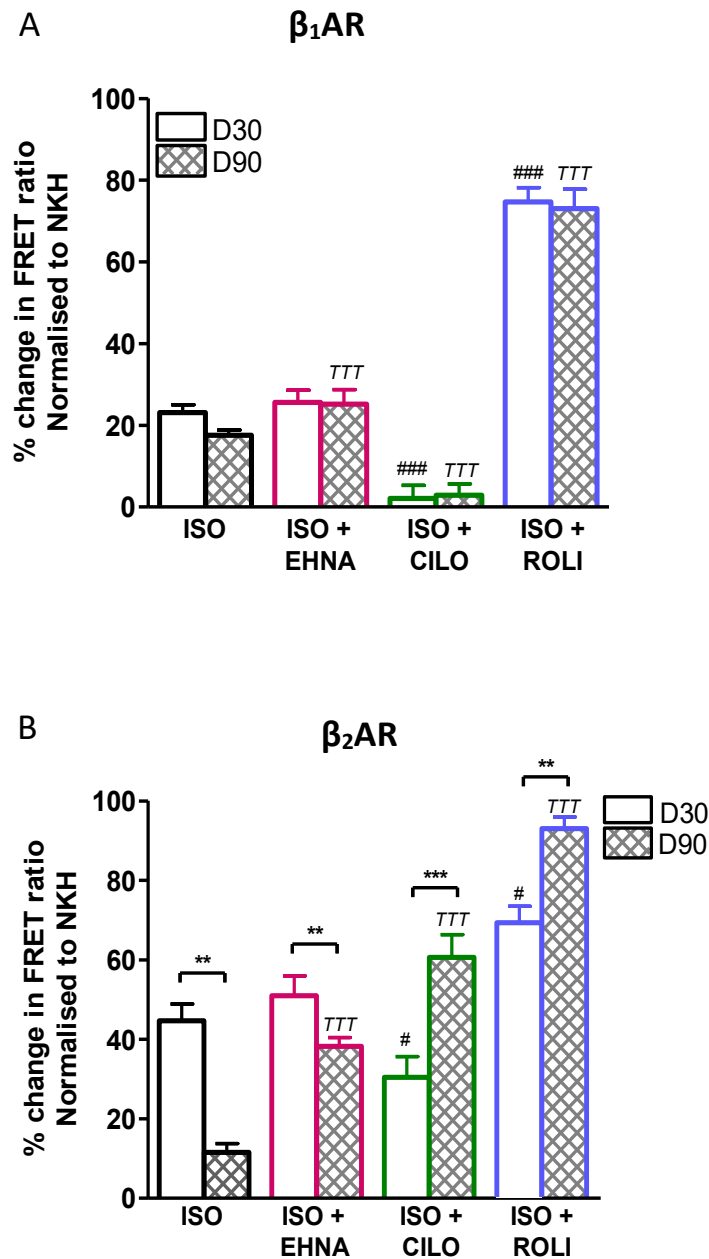


Figure 6. 17 Aged iPSC-CMs shows compartmentalised β_2AR -cAMP and PDE2, 3 and 4

A) cAMP degrading PDE activity associated with β_1AR and B) β_2AR was assessed in iPSC-CMs at D30 and D90 with isoprenaline in the presence of ICI or CGP respectively, and subsequent inhibition of PDE2, PDE3 or PDE4 with EHNA, cilostamide or rolipram. The percentage change in FRET ratio was normalised to maximal cAMP produced upon AC activation with NKH. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test. ** $p < 0.01$, *** $p < 0.001$. # = significant from D30 ISO, T = significant from D90 ISO

6.3.14 Summary of PDE2, PDE3 and PDE4 activity towards β AR-cAMP pools

Based on the basal tones described in sections 6.13-15, a comparison of PDE2, 3, and 4 with and without caveolae was made.

Upon comparison of β_1 AR-dependent responses, PDE2 tone was not influenced by caveolae removal, nor did maturation alter PDE2 response on cytosolic cAMP. PDE3 responses were significantly lower than both PDE2 and PDE4 activity ($p < 0.05$); most likely as an opposing response of PDE3 on cAMP was noted. Inhibition of PDE3 resulted in a decrease in cAMP. The removal of caveolae resulted in a more significant decrease in cAMP ($p < 0.05$), however, this was only significant in D30 cells and to a smaller extent in D90 cells. In comparison to PDE2 and PDE3, PDE4 had the greatest influence on cAMP degradation, and even more so after iPSC-CMs were aged. The removal of caveolae had no influence on PDE4 activity in early or late stage iPSC-CMs.

Similarly, β_2 AR-dependent responses of PDE2, 3, and 4 were assessed. Interestingly, unlike β_1 AR-dependent response to PDE2 activity, β_2 -dependent response was sensitive to caveolae removal and a significant decrease in PDE2 activity was noted ($p < 0.05$). Although a reduced level of PDE2 activity was noted in aged iPSC-CMs, the response was not significant. Furthermore, caveolae removal didn't affect PDE2 activity. Similar to the response of β_1 AR, β_2 AR-dependent PDE3 response was significantly lower than that of PDE2 and PDE4 ($p < 0.05$). PDE3 inhibition seems to be decreasing cytosolic cAMP. Removal of caveolae had no effect on PDE3 activity in early iPSC-CMs, however, interestingly, PDE3 activity was abolished in aged cells. The level of PDE4 activity on β_2 AR-cAMP seems similar to PDE2 activity, unlike on β_1 AR-cAMP pools, and this level of activity was not altered as cells were aged. Interestingly, PDE4 activity was enhanced after caveolae removal in aged cells (Fig 6.18).

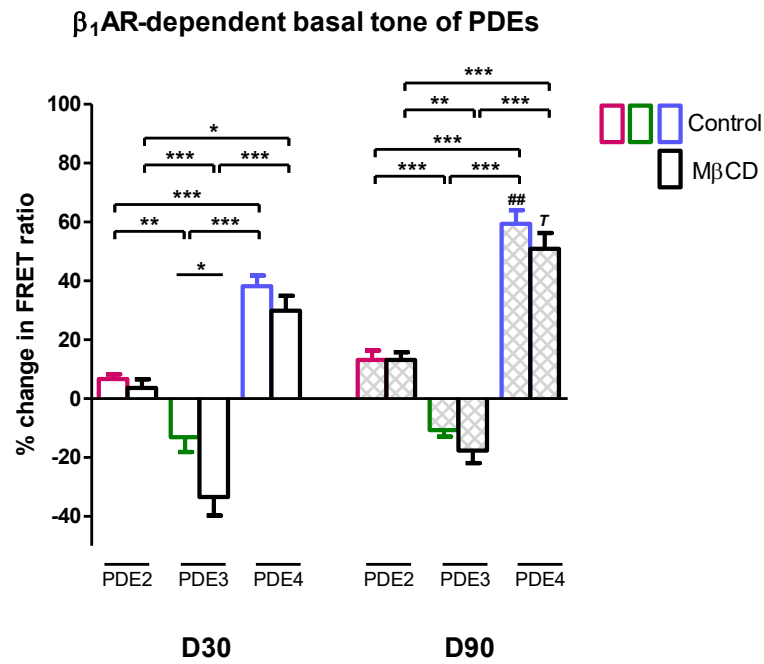
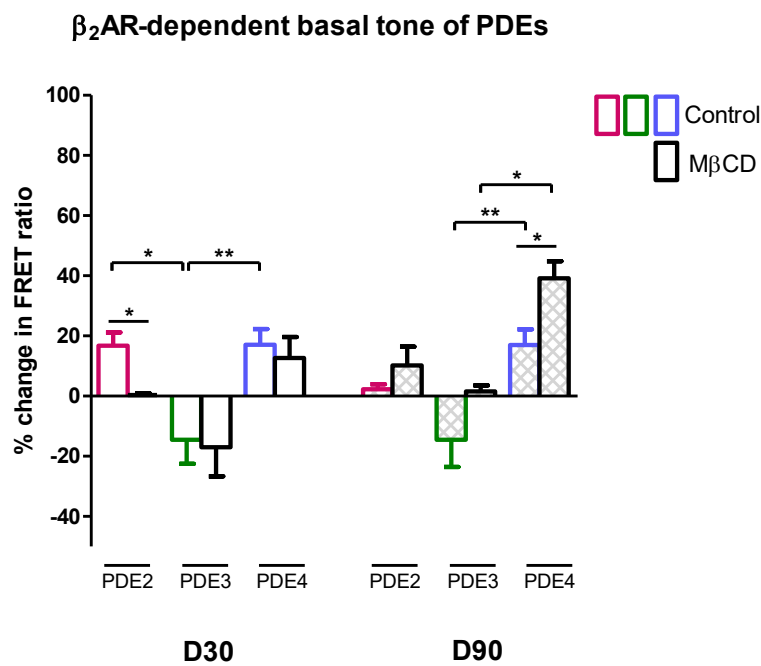


Figure 6. 18 Summary of PDE2, 3 and 4 tone upon β AR-cAMP in caveolae compartmentation

An overall comparison of the basal tones of PDE2, 3 and 4 activity associated with β_1 AR and β_2 AR in iPSC-CMs at D30 and D90 in the presence or absence of caveolar microdomains. Data are presented as mean $\pm$ SEM. Significance was determined by One-Way ANOVA with Bonferroni post-hoc test.

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

6.4 DISCUSSION

In this chapter the effect of prolonging iPSC-CMs culture time from 30 days to 90 days as a method of maturation was investigated by studying the functional developments of the β AR pathway. Changes in gene expression of the β AR signalling pathway components were assessed, along with the structural development of caveolae, and their role on compartmentalising β ARs-cAMP pools. Furthermore, the gene expression levels of PDE2, 3, and 4 and their functional activity was investigated as iPSC-CMs are aged.

α -Actinin and cTnT are an integral part of the contractile machinery and are strong markers of cardiac phenotype. Their expression levels are known to increase throughout iPSCs differentiation along the cardiac lineage. Their expression levels are shown to occur early in iPSC-CMs (by D30) (Cai et al., 2019), which supports this study; robust expression of both α -actinin and cTnT is seen by D30 and no further increase in expression was observed as cells were aged. This indicates that these proteins are already robustly expressed in early cardiomyocytes and ageing alone does not enhance expression levels. To develop a deeper insight into the functional relevance of these proteins, it would be important to investigate their orientation and alignment, as well as interaction with other proteins essential within the sarcomeric complex. Previous studies report that long term culture of iPSC-CMs (45 days) significantly increases thin filament, and therefore, sarcomere length, and also increased fibre density, definition and alignment (Dias et al., 2018; Lundy et al., 2013a); although expression levels remain lower than that of the adult left ventricle (Kamakura et al., 2013).

Along with α -actinin and cTnT of the sarcomere, Bin1, Jph2 and CAV3 are crucial proteins of the T-tubular network. The effect of prolonged culture of iPSC-CMs on these proteins or T-tubular development has not previously been documented. However, one previous study has reported through transcriptome analysis that cardiac tissue formed from early-stage iPSC-CMs subject to electromechanical conditioning along with four weeks in culture increases Bin1, Jph2 and CAV3

expression (Ronaldson-Bouchard et al., 2018b). In this study we find that there was a slight reduction in Bin1 and no overall change in Jph2 gene expression. Previous studies show there is an increase in expression of CAV3 from D30 to D90 (Gintant et al., 2019; Jung et al., 2016; Ronaldson-Bouchard et al., 2018b). In this study, this correlates well with the increase in CAV3 density observed in aged cells. CAV3 induces the formation of caveolae, which are well known to play an important role in the development of T-tubules. This report found maturation of caveolae in aged iPSC-CMs, reflected by an increase in CAV3 gene expression, protein density and membrane caveolae by D90.

The functional consequence of membrane caveolar structures in compartmentalising β_2 ARs as iPSC-CMs are aged was then investigated by using M β CD to remove membrane cholesterol, hence disrupting caveolae compartments. In comparison to the results in the previous chapter where early stage iPSC-CMs were characterised, caveolae removal in aged iPSC-CMs significantly augmented β AR-cAMP pools. This is an important indication that not only are caveolae membrane domains increasing in density by ageing iPSC-CMs, but are also functionally relevant as they are restricting β AR signalling. It is interesting to note that M β CD treatment augments β_2 AR- signalling more extensively than it does β_1 AR-dependent cAMP signalling.

To further depict the maturation of the β AR signalling system, the gene expression of β_1 AR and β_2 AR were first compared in early (D30) and late (D90) stage iPSC-CMs. β_2 ARs are known to be the dominant receptor subtype in early myocytes (Jung et al., 2016; Haodi Wu et al., 2015), as is found in this study. However, previous literature reports a dramatic increase in β_1 AR expression as iPSC-CMs are aged in culture with an expression level several folds higher than that of β_2 AR. This does not compare with the results of this study, as β_2 AR remains to be the dominating receptor here. This highlights the variability we face in iPSC-CMs studies possibly due differences in the differentiation protocols used, culture conditions and media, or the genetic background of different cell lines.

This study continued by exploring the functional responses of the β AR subtype-specific signalling as responses to agonists stimulation are an indicator of phenotypic maturation (Huebsch et al., 2016), and has been indicated to be developmentally regulated (Germanguz et al., 2011). The β AR subtype specific responses to isoprenaline were detected by FRET microscopy, with the EC50 values increasing from 4.8nM and 2.4nM at D30 to 14.4nM and 7.7nM at D90 for β_1 AR and β_2 AR respectively. In previous studies, subtype-specific responses were not identified, however the overall EC50 of total β ARs in iPSC-CMs at D12 of culture had similar EC50 value of 14nM observed by beat frequency, which is also known to correspond to that observed in clinical studies (Ronaldson-Bouchard et al., 2018b). This indicates that ageing iPSC-CMs of this cell line is having a positive influence on receptor function as it presents a similar magnitude of response to those of clinical studies, thus displaying similar pharmacological responsiveness.

Functional regulation by β AR signalling in iPSC-CMs has most commonly been conducted in previous studies by assessment of calcium handling properties and chronotropic or inotropic activity, which are the end-stage responses upon receptor stimulation. However, the knowledge of immediate cellular responses to receptor activation through cAMP activity is limited in age-matured cells. Using FRET microscopy to measure real-time cAMP output, β_1 AR and β_2 AR receptor response and receptor subtype-dependent PDE activity was assessed in aged iPSC-CMs for the first time. The role of β_1 AR signalling remained rather constant throughout ageing of iPSC-CMs as cAMP output upon receptor stimulation was consistent. Interestingly, despite β_2 AR expression increasing with time, its functional role decreased. The discrepancy between expression level and functional activity may be due to the way the response is processed in the cell. For example, there is indication from previous literature that during the late-stages of iPSC-CM maturation, there is a loss of β_2 AR coupling to G_s proteins due to reduced G_s expression and increased G_i expression throughout maturation (Haodi Wu et al., 2015). This would require further investigation in this cell line in future studies. On the other hand, the localisation of the receptors themselves may be an explanation. As described above, ageing iPSC-CMs

allows maturation of caveolar compartments, which may be involved in the physical compartmentation of the β_2 ARs, thus encouraging local pools of cAMP and reducing cytosolic cAMP levels.

Further to this, cAMP handling through PDE activity may play an essential role in compartmentalising receptor signalling and may lead to milder responses to β AR stimulation as iPSC-CMs are matured at later stages. The overall activity of PDE on β_1 AR and β_2 AR remained consistent in early and aged iPSC-CMs as non-selective PDE inhibition regulated the same amount of cAMP degradation. Deeper investigation into the PDE families revealed that PDE4D was most significantly expressed; although, ageing did not affect PDE expression levels. Contradictory to this, an increase in many PDE family proteins in matured iPSC-CMs has been previously reported, thus supporting the suppression of cAMP signalling (Haodi Wu et al., 2015). This would require further investigation in this cell line, and potentially the use of other techniques for PDE detection.

Although this report shows that the expression levels of PDEs were not altered as iPSC-CMs were aged, Wu and colleagues observed contradictory findings. They show that through maturation of iPSC-CMs, PDE2A expression levels are significantly lowered by D12 and remains low throughout maturation. By D60, PDE2A expression levels of iPSC-CMs are similar to that of left ventricular (LV) myocytes. PDE3A expression is significantly increased by D60, although this level of expression remains substantially lower than the expression level of the LV myocytes. Furthermore, they also show that PDE4 expression is significantly raised by D30 and continues to increase by D60, at which point its expression level is comparable to the LV myocytes (H Wu et al., 2014). This provides valuable insight into how PDE expression levels are altered as iPSC-CMs are aged, however, further to this, how changes in the functional activity or cellular localisation may have developed were not investigated.

This report investigated this further through the use of selective inhibitors during the FRET protocols for real-time cAMP assessment. PDE2 is known to be tightly coupled to the pool of ACs which are

activated upon β AR stimulation (Mongillo et al., 2006), and therefore plays a critical role in modulating β AR-cAMP. Results here show that prolonging culture time of iPSC-CMs reduces PDE2 activity towards β_2 AR-cAMP pools but not β_1 AR-cAMP. This supports previous literature which also shows an overall reduction in PDE2 expression levels as iPSC-CMs were aged, although receptor subtype specificity was not highlighted. Moreover, PDE2 activity has been noted to increase significantly in a DCM iPSC-CMs and human heart failure (Mehel et al., 2013; H Wu et al., 2014). This may indicate that PDE2 activity is lowered during the developmental stages of iPSC-CMs, and enhanced in disease states. The upregulation of PDE2 during disease plays a protective role against cardiac stress by desensitising against excessive β AR drive, and blunting receptor responses.

The presence of PDE2 has been previously suggested to be in the cytosol, mitochondria and also at the plasma membrane in human atrial myocytes, given its association with β AR-cAMP pools (Guellich et al., 2014; Pavlaki & Nikolaev, 2018). However, its specificity towards β_1 AR at the non-caveolar membrane or β_2 AR at caveolae membrane has not previously been identified. Results from this chapter show that PDE2 activity did not seem to change towards β_1 AR-cAMP pools upon disruption of caveolae. However, its activity based on β_2 AR-cAMP in early iPSC-CMs was variable in the presence and absence of caveolae. This may suggest that PDE2 is influenced by β_2 AR-cAMP and is sensitive to signalling compartmentation. At this stage, it is difficult to elucidate the exact localisation of PDE2 as further experimentation can help clarify these findings. In particular, the use of a localised FRET sensor would be of utmost importance. Furthermore, PDE2 is also sensitive to cGMP, thus, can also influence PDE2 regulation. So, an assessment of the background cGMP in iPSC-CMs would also need to be considered.

PDE3 contributes to cAMP hydrolysis in various intracellular compartments of the human myocardium (Hambleton et al., 2005b). Previous literature suggests that PDE3 isoforms localise to both the cytosolic and particulate fraction (Guellich et al., 2014), with PDE3A being localised to the sarcoplasmic reticulum, and PDE3B to caveolin3-rich regions of the plasma membrane, such as the T-tubules, and

in close proximity to mitochondria, suggesting that it may also play a role towards regulating energy metabolism (Movsesian et al., 2018). Their association with the individual subtypes of β AR-cAMP is not yet clear. This study finds that PDE3 activity is not altered during iPSC-CMs maturation, nor show any particular association with the β_1 AR or β_2 AR. However, PDE3 inhibition significantly changes cyclic nucleotide handling in iPSC-CMs in comparison to PDE2 and PDE4. It shows that upon β ARs stimulation, PDE3 inhibition reduced cytosolic cAMP concentration, suggesting that it may be functioning against cAMP degradation or for maintenance of basal cAMP. Although previous literature highlights that PDE3 inhibitors have a protective role, it is actually used clinically to increase contractility in patients with heart failure (Movsesian et al., 2018). Furthermore, it is also indicated that the reduction of PDE3A enhances cardiomyocyte apoptosis by upregulating proapoptotic transcription factors (Ding et al., 2005). This suggest, PDE3 inhibition functions towards increasing cAMP concentrations, thus enhancing myocyte contractility in the short-term, but can increase cardiovascular mortality on their long-term administration. The combination of beneficial versus adverse effects of PDE3 inhibition presents a challenge in understanding the responses seen in iPSC-CMs. The response of PDE3 inactivation in may have opposing effects on cAMP handling in different subcellular compartments which need to be further explored.

PDE4 isoforms are known to control PKA-mediated regulation of β_1 ARs and β_2 ARs (Richter et al., 2011). A previous study shows that inhibition of PDE4 in human hearts leads to increased phosphorylation of a restricted number of proteins in comparison to rodent myocytes where almost all PKA substrates are phosphorylated. Therefore, PDE4 inhibition does not have 'global' effects on cAMP signalling (Richter et al., 2011). Inactivation of PDE4 has been shown to potentiate inotropic responses to catecholamines or PDE3 inhibition (Surdo et al., 2017) , suggesting that PDE4 is a crucial player under conditions of elevated cAMP. The role of PDE4 towards β AR subtypes has not been previously elucidated, and was a core part of this chapter. We find that there is a substantial role of PDE4 towards β_1 AR-cAMP pools at both early and late stages of iPSC-CMs maturation with no detectable change in

activity after caveolae removal. This is as expected, as the majority of β_1 AR are localised in non-caveolae regions of the cells and so are not influenced by disruption of membrane compartmentation. Furthermore, it supports the previous studies as its activity in age-matured cells is becoming more prominent and is responsible for a greater proportion of cAMP degradation as discussed above.

PDE4 activity towards β_2 AR-cAMP is less pronounced than β_1 AR-coupled responses, indicating PDE4 plays a smaller role in cAMP degradation in localised compartments. Interestingly, the disruption of cellular microdomains enhanced PDE4 activity in aged cells. This indicates that PDE4 activity is enhanced upon increases in cytosolic cAMP when it is no longer confined to local membrane pools. Furthermore, the change in enzyme activity upon ageing indicates that changes to PDE4 localisation may occur during the maturation process of iPSC-CMs. Similar studies need to be conducted in adult myocytes to make comparisons of PDE4 activity and strengthen our current understanding of PDE4 activity in various subcellular localisations. In particular, PDE4 also exists in various isoforms, and so how isoform expression and activity changes in subcellular compartments needs to be further assessed.

Limitations and future work

To develop a deeper understanding of signalling compartmentation, membrane specific sensors would prove more beneficial as they will be able to detect local cAMP pool with greater accuracy and sensitivity. This study utilised a cytosolic FRET sensor which may only have a limited capacity for detecting localised cAMP pools. One previous study depicts the use of Cutie sensors which can be targeted to specific cellular domains, thus allowing the study of cyclic nucleotides at the nanodomain level (Surdo et al., 2017).

To further support this study, a comparison to adult human cardiomyocytes or tissue would strengthen these findings. To understand whether the method of ageing iPSC-CMs in culture is an

effect method of cellular maturation, the experiments conducted above should also be replicated in mature adult cells. This would allow a more appropriate comparison of whether the desired signalling outcome is achieved. However, this is not to say that the findings here are nullified, as they remain true to this particular cell line under the outlined culture and treatment conditions. Real-time studies of β AR and PDE based cAMP are limited, so this study is the first to provide such insights. It may also be supportive of this study to have been conducted in other iPSC or hESC cell lines to indicate whether similar outcomes are achieved, therefore proving more robust.

One crucial functional property of β ARs is the phenomenon of desensitisation which can occur through receptor internalisation, receptor phosphorylation, PKA phosphorylation or receptor downregulation. To fully understand the β AR pathway and its maturation status, further investigations into these mechanisms is essential.

Finally, though ageing iPSC-CMs has shown, through this study and those before it, that it has a positive influence on major structural and functional properties, it has limited throughput and is resource and time inefficient. The effect of CAV3-overexpression conducted in this study also displays a positive influence on caveolae microdomain development and β AR signalling. This could potentially be one alternative method to ageing to be explored. It would significantly reduce time as well as cost of culture resources. Also, it would also prevent any dedifferentiation of iPSC-CMs which could occur with increased culture time.

Summary

In this chapter we find that age-dependent maturation of iPSC-CMs from 30- to 90-days in culture enhances CAV3 gene and protein expression which significantly promotes membrane caveolae development. Prolonging culture time also increases β AR levels. Despite increased gene expression of β_2 ARs, we see a decrease in its functional activity. This is as would be expected of mature adult myocytes; an increase in caveolae number would signify greater compartmentation of the β_2 ARs, thus correlating with a decrease in cytosolic cAMP. This indicates a substantial method of maturation of iPSC-CM microdomain development. Furthermore, we see altered functional activity as iPSC-CMs are aged, along with difference regarding association with caveolae microdomains. Therefore, this chapter supports the hypothesis that enhancing the natural developmental process of cardiac microdomains will positively influence on β AR-cAMP signalling activity. However, due to the use of a cytosolic FRET sensor, caveolae compartments were not thoroughly depicted. In addition, the lack of PDE-isoform blockers, only the overall families could be investigated. Future work should make use of localised FRET sensors, and also incorporate other methodologies to assess PDE localisation, such as blotting with cell fractionation.

CHAPTER 7: FINAL DISCUSSION AND CONCLUSIONS

The primary aim of this thesis was to develop a deeper insight into the β_2 ARs functional activity in stem-cell derived myocytes and to use their signalling activity as a prototype to investigate maturation techniques of the iPSC-CMs. The role of caveolae as functional signalosomes with respect to β ARs activity in iPSC-CMs and its influence on compartmentalising cAMP-degrading PDEs was assessed.

The thesis initially studied the 4 most common β_2 AR haplotypes in the human population to lay the foundations of the receptor's functional properties. It further highlighted the extent of biased agonism as a protective mechanism during induced catecholaminergic stress through receptor desensitisation. Through assessing cAMP activity and chronotropic responses, the differential activity of each haplotype was identified, thus indicating the influence of genetic variations on health and disease, and their importance during drug administration. This study reports evidence of β_2 AR- G_i interaction in hESC-CMs upon prolonged stimulation; however, it also indicates that there are other protective mechanisms of action in play. Receptor internalisation or downregulation are two possible mechanisms which need to be explored further (Rajagopal & Shenoy, 2018). Furthermore, β_1 ARs also influence cAMP signalling and chronotropic activity of the cells (Nikolaev et al., 2006). Unsurprisingly, this gene also contains various SNPs which are present in the population (Liggett, 2010). It is important for further studies to consider any variants which may be in linkage disequilibrium with the β_2 AR variants, and this provide further indication of phenotypic response. Understanding of various physiological properties of β_2 ARs would not have been possible without the use of cell models, which have relied heavily on the CRISPR/Cas9 gene editing technique, and without which, the data output may not have been accurate or specific.

One of the most commonly described mechanisms of SC-CMs maturation is via direct structural manipulation (Kijlstra et al., 2017; Lehnert et al., 2004; Mcdevitt et al., 2002; Ribeiro et al., 2015; Salick et al., 2014). This was explored through microcontact printing in this thesis, and how structural maturation affects β_2 AR functional activity was investigated through measuring cellular cAMP and its compartmentation by PDEs. A significant improvement on cell geometry was noticed. As opposed to

the normal, unstructured iPSC-CMs, microcontact printing allowed cells to be patterned, thus taking on any desired shape. In particular, patterning them onto fibronectin lanes of known width showed dramatic improvements in cell shape and structural protein alignment. Improvements in sarcomeric organisation are known to indicate improved contractile activity. Though this was not studied in this project, it would be of significant benefit to explore calcium handling and other functional properties of patterned cells to build on current knowledge (Bray et al., 2008b; Kijlstra et al., 2017; Kuo et al., 2012b; Ribeiro et al., 2015; Silbernagel et al., 2020). Nonetheless, the changes in β_2 AR-cAMP handling and PDE activity were explored as an assessment of improvements in functional output. Unfortunately, despite positive influences on structural maturation, complementary functional improvements were not as dramatic/defined. Little change was observed in caveolae content, β_2 AR signalling activity and PDE responses. This is a strong reminder that despite progress being reported in multiple aspects of iPSC-CMs development and maturation, studies should actively focus on studying improvements in a combination of both structural and functional aspects.

Structural manipulation of cell shape alone was not able to generate the desired change in β_2 AR signalling properties as initially expected, so this project diverted its focus towards development of microdomain compartmentation. In early myocytes, at the age of day 30 from the start of differentiation, a fair proportion of CAV3 gene and protein expression could be detected. This raised the question whether caveolae in iPSC-CMs play the role of signalosomes as had been recorded in mature cells in previous literature (Ravi C. Balijepalli & Kamp, 2008; Calaghan et al., 2008; Calaghan & White, 2006a; Harvey & Calaghan, 2012; MacDougall et al., 2012; Rybin et al., 2000). In particular, compartmentation of the β_2 ARs was of initial interest. The localisation of β_2 ARs within caveolae was noted in the IMR iPSC-CMs cell lines. This posed an interesting observation, and indication that one line may reflect a more mature phenotype. We decided to continue the remainder of the study on the less mature cell line to study methods of maturation of β_2 AR signalling and compartmentation via caveolar microdomains. In early myocytes, little effect of cAMP degrading PDE2, 3 and 4 were noted

on β_1 AR-cAMP pools. Interestingly, PDE2 and 4 seemed to be more greatly involved in β_2 AR-cAMP pool degradation. Further investigation into the specific cellular localisation of these cAMP pools are of primal importance, and should be investigated through the use of localised FRET sensors and protein analysis upon cell fractionation (Surdo et al., 2017).

A method of cellular ageing was investigated as a method of iPSC-CMs maturation as previously described (Dias et al., 2018; Jung et al., 2016; Kamakura et al., 2013; Lewandowski et al., 2018); cell culture was prolonged from 30 days to 90 days. Interestingly, gene expression and protein density were both significantly enhanced. This positive response also led to increased caveolar microdomains, similar to that seen by overexpressing CAV3. Interestingly, despite increased β_2 AR gene expression, its functional response had declined by day 90. This is a significant indication of compartmentation of receptor and its associated signalling. The process of this relocalisation was not investigated in this study and is not fully defined in previous literature and remains under investigation (Vistein & Puthenveedu, 2013). This would be of great importance to depict. Further to this, prolonging cell culture plays a dynamic role in PDE activity. To form a firmer conclusion of PDE localisation, it would be of great importance to use membrane localised FRET sensors to depict activity in specific cellular microdomains (Surdo et al., 2017). Furthermore, studying individual PDE activity using specific inhibitors may not reveal the whole picture. In particular, as many recent studies are beginning to understand the importance of cyclic nucleotide cross-talk (Pavlaki & Nikolaev, 2018; Zaccolo & Movsesian, 2007; C. Y. Zhao et al., 2016). cAMP and cGMP are maintained at nanomolar concentrations in specific cellular domains. Disruption to this equilibrium in one domain will no doubt have implications in other cellular domains. This has not been investigated in this study, but unveiling the cross-talk of cyclic nucleotides in neighbouring microdomains will reveal significant depths of signalling pathways.

Limitations and future work

This project used a cytosolic FRET sensor to detect changes in cAMP concentrations in the cell upon β_1 AR or β_2 AR stimulation. Although changes in cAMP concentration were detected upon various drug treatments, a more accurate response of cAMP activity could have been achieved by using a localised FRET sensor. More recently, FRET sensors which can be localised to various compartments of the cells have been developed. These allow the study of cAMP in specific subcellular localisations. This would have been a great tool to use in this study and should be considered in future work.

cAMP signalling was the key cyclic nucleotide of this project. However, its activity can be significantly influenced by cGMP. There are emerging studies where the cross-talk between cAMP and cGMP are highlighted. A limitation of this study is that background activity of cGMP and its influence on cAMP signalling was not monitored. This would require the use of a dual FRET sensor, making use of four fluorophores which could detect the change in both cAMP and cGMP simultaneously. Furthermore, if these could be targeted to specific membrane domains, the interaction between the two nucleotides could be studied more effectively. It may also be able to provide how changes in one nucleotide can affect the other in neighbouring microdomains.

To study PDE activity on cAMP degradation, family-specific inhibitors were used throughout the FRET experiments. This study was limited to only studying PDE families as a full set of subtypes- or isotype-specific inhibitors are not available. There are numerous isoforms of PDEs which exist in cardiomyocytes, and can function in various domains. Therefore, this study is not clearly able to distinguish between the activity of the PDE subtypes. One way to partially overcome this limitation is through conducting a western blot after cell fractionation. PDE subtypes could be blotted against based on the isolated regions of the cell to give an indication of the residing PDEs and their protein density. However, this may not correlate with PDE functionality and still would need to be investigated in other ways. Furthermore, this project has not yet been able to depict PDE-crosstalk. Due to the vast

range of PDEs, it would not be surprising that the inhibition of one PDE would lead to the activation of another through a compensatory mechanism. This would also require further investigation.

Maturation of iPSC-CMs through prolonged culture time is an effective mechanism of enhancing microdomain development. However, 3-month culture times are not time- or resource- efficient. Understanding the changes which are taking place during these three months, and enhancing such processes may overcome the time limitation. Furthermore, as shown in Chapter 5, CAV3 overexpression may also prove beneficial to some extent as this also revealed microdomain development. This could be combined with patterning iPSC-CMs as a method of developing structural and functional maturation.

During the initial parts of Chapter 5, two iPSC-CMs cell lines were used for exploring caveolae components and β AR signalling. As the chapter progressed, the IMR cell line was selected for conducting the remainder of the work as it showed relatively less maturity with respect to β_2 AR-cAMP compartmentation. As described in previous literature, variations in phenotypic properties exist between line to line. Therefore, this prevents direct comparative studies from being performed between studies. Using only a single cell line in this project is one of the limitations. Similar experiments should be conducted in other iPSC-CMs cell line to strengthen these findings. Furthermore, adult human cells could also be used as a comparison.

SICM and patch clamp techniques could only be briefly conducted on control cells in this project without investigating the influence on LTCC activity in matured cells. This preliminary confirms the success of these techniques on iPSC-CMs and gives insight into the activity of LTCCs. Future work is required to focus on how MP treatment may influence LTCC, particularly in the deepening invaginations which were detected after MP treatment. As aged cells displayed increased caveolae density and β_2 AR re-localisation, it may be possible that ageing cells also display LTCC compartmentation; this should also be studies using patch clamp techniques.

One crucial component of myocyte function is Ca^{2+} handling for the regulation of contraction, but was not explored in this project. Future work should also consider how age-dependent maturation may influence other downstream effector molecules of the βAR signalling pathway. Patterning iPSC-CMs indicates a promising contractile output due to their isometric contractile properties. As patterning iPSC-CMs shows an improved structural phenotype in Chapter 4, the inotropic and chronotropic function would also provide a good indication of Ca^{2+} handling and hence functional maturation.

In conclusion, this thesis accepts the hypothesis that $\beta_2\text{AR}$ genetic variants play an important role in regulation of receptor-dependent cAMP signalling due to their variable G_s/G_i composition, thus, influencing cardiomyocyte physiology. It also accepts that iPSC-CMs possess limited and underdeveloped structural microdomains, thus displaying an immature and uncoordinated $\beta_2\text{AR}$ -derived cAMP signalling and PDE activity. It accepts that employing a microcontact printing mechanism to encourage controlled, structured growth does enhance the structural development, although it rejects that this alters $\beta_2\text{AR}$ -cAMP signalling. However, it accepts that the process of cardiac microdomains development though aging alters $\beta_2\text{AR}$ -dependent cAMP signalling and PDE activity.

REFERENCES

- Agarwal, S. R., MacDougall, D. A., Tyser, R., Pugh, S. D., Calaghan, S. C., & Harvey, R. D. (2011). Effects of cholesterol depletion on compartmentalized cAMP responses in adult cardiac myocytes. *Journal of Molecular and Cellular Cardiology*, 50(3), 500–509. <https://doi.org/10.1016/j.yjmcc.2010.11.015>
- Ahles, A., & Engelhardt, S. (2009). Polymorphisms determine β -adrenoceptor conformation: implications for cardiovascular disease and therapy. *Trends in Pharmacological Sciences*, 30(4), 188–193. <https://doi.org/10.1016/j.tips.2009.02.001>
- Ahmad, F., Murata, T., Shimizu, K., Degerman, E., Maurice, D., & Manganiello, V. (2015). Cyclic nucleotide phosphodiesterases: Important signaling modulators and therapeutic targets. *Oral Diseases*, 21(1), e25–e50. <https://doi.org/10.1111/odi.12275>
- Alaiti, M. A., Ishikawa, M., & Costa, M. A. (2010). Bone marrow and circulating stem/progenitor cells for regenerative cardiovascular therapy. In *Translational Research* (Vol. 156, Issue 3, pp. 112–129). Mosby Inc. <https://doi.org/10.1016/j.trsl.2010.06.008>
- Allen, J. A., Halverson-Tamboli, R. A., & Rasenick, M. M. (2007). Lipid raft microdomains and neurotransmitter signalling. In *Nature Reviews Neuroscience* (Vol. 8, Issue 2, pp. 128–140). <https://doi.org/10.1038/nrn2059>
- Amin, A. S., Tan, H. L., & Wilde, A. A. M. (2010). Cardiac ion channels in health and disease. *Heart Rhythm*, 7(1), 117–126. <https://doi.org/10.1016/j.hrthm.2009.08.005>
- Armour, J. A., Murphy, D. A., Yuan, B., MacDonald, S., & Hopkins, D. A. (1997). Gross and microscopic anatomy of the human intrinsic cardiac nervous system. *The Anatomical Record*, 247(2), 289–298. [https://doi.org/10.1002/\(SICI\)1097-0185\(199702\)247:2<289::AID-AR15>3.0.CO;2-L](https://doi.org/10.1002/(SICI)1097-0185(199702)247:2<289::AID-AR15>3.0.CO;2-L)
- Atmanli, A., & Domian, I. J. (2013). Generation of aligned functional myocardial tissue through microcontact printing. *Journal of Visualized Experiments : JoVE*, 73. <https://doi.org/10.3791/50288>
- Baillie, G. S. (2009). Compartmentalized signalling: spatial regulation of cAMP by the action of compartmentalized phosphodiesterases. *FEBS Journal*, 276(7), 1790–1799. <https://doi.org/10.1111/j.1742-4658.2009.06926.x>
- Baker, J. G. (2005). The selectivity of β -adrenoceptor antagonists at the human β_1 , β_2 and β_3 adrenoceptors. *British Journal of Pharmacology*, 144, 317–322. <https://doi.org/10.1038/sj.bjp.0706048>
- Baker, J. G. (2010). The selectivity of β -adrenoceptor agonists at human β_1 -, β_2 - and β_3 -adrenoceptors: RESEARCH PAPER. *British Journal of Pharmacology*, 160(5), 1048–1061. <https://doi.org/10.1111/j.1476-5381.2010.00754.x>
- Balijepalli, R C, Foell, J. D., Hall, D. D., Hell, J. W., & Kamp, T. J. (2004). Localization of cardiac L-type Ca^{2+} channels to a caveolar macromolecular signaling complex is required for β_2 -adrenergic regulation. In *J. Gen. Physiol* (Vol. 124, Issue 19). www.pnas.org/cgi/doi/10.1073/pnas.0503465103
- Balijepalli, Ravi C., Foell, J. D., Hall, D. D., Hell, J. W., & Kamp, T. J. (2006a). Localization of cardiac L-type Ca^{2+} channels to a caveolar macromolecular signaling complex is required for β_2 -adrenergic regulation. *Proceedings of the National Academy of Sciences of the United States of America*, 103(19), 7500–7505. <https://doi.org/10.1073/pnas.0503465103>
- Balijepalli, Ravi C., & Kamp, T. J. (2008). Caveolae, ion channels and cardiac arrhythmias. In *Progress in Biophysics and Molecular Biology* (Vol. 98, Issues 2–3, pp. 149–160). <https://doi.org/10.1016/j.pbiomolbio.2009.01.012>

- Balijepalli, Ravi C., Lokuta, A. J., Maertz, N. A., Buck, J. M., Haworth, R. A., Valdivia, H. H., & Kamp, T. J. (2003). Depletion of T-tubules and specific subcellular changes in sarcolemmal proteins in tachycardia-induced heart failure. *Cardiovascular Research*, 59(1), 67–77. [https://doi.org/10.1016/S0008-6363\(03\)00325-0](https://doi.org/10.1016/S0008-6363(03)00325-0)
- Beavo, J., & Houslay, M. (1990). *Cyclic nucleotide phosphodiesterases: Structure, regulation and drug action*.
- Beavo, JA, Francis, S., & Houslay, M. (2006). *Cyclic nucleotide phosphodiesterases in health and disease*. <https://books.google.com/books?hl=en&lr=&id=Q0fMBQAAQBAJ&oi=fnd&pg=PP1&ots=8xUI4FOuH0&sig=QIIKRk0jYwM3wtvjcptkEJxZoo>
- Bedada, F. B., Wheelwright, M., & Metzger, J. M. (2016). Maturation status of sarcomere structure and function in human iPSC-derived cardiac myocytes. In *Biochimica et Biophysica Acta - Molecular Cell Research*. <https://doi.org/10.1016/j.bbamcr.2015.11.005>
- Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heide, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H., Jovinge, S., & Frisén, J. (2009). Evidence for cardiomyocyte renewal in humans. *Science*, 324(5923), 98–102. <https://doi.org/10.1126/science.1164680>
- Bers, D. M. (2002). Cardiac excitation-contraction coupling. In *Nature* (Vol. 415, Issue 6868, pp. 198–205). Nature Publishing Group. <https://doi.org/10.1038/415198a>
- Bers, D. M. (2008). Calcium Cycling and Signaling in Cardiac Myocytes. *Annual Review of Physiology*, 70(1), 23–49. <https://doi.org/10.1146/annurev.physiol.70.113006.100455>
- Bers, D. M., & Despa, S. (2009). Na/K-ATPase-An Integral Player in the Adrenergic Fight-or-Flight Response. In *Trends in Cardiovascular Medicine* (Vol. 19, Issue 4, pp. 111–118). Elsevier. <https://doi.org/10.1016/j.tcm.2009.07.001>
- Bethani, I., Skånland, S. S., Dikic, I., & Acker-Palmer, A. (2010). Spatial organization of transmembrane receptor signalling. *The EMBO Journal*, 29(16), 2677–2688. <https://doi.org/10.1038/emboj.2010.175>
- Bhargava, A., Lin, X., Novak, P., Mehta, K., Korchev, Y., Delmar, M., & Gorelik, J. (2013). Super-resolution scanning patch clamp reveals clustering of functional ion channels in adult ventricular myocyte. *Circulation Research*, 112(8), 1112–1120. <https://doi.org/10.1161/CIRCRESAHA.111.300445>
- Binah, O., Dolnikov, K., Sadan, O., Shilkrut, M., Zeevi-Levin, N., Amit, M., Danon, A., & Itskovitz-Eldor, J. (2007). Functional and developmental properties of human embryonic stem cells-derived cardiomyocytes. *Journal of Electrocardiology*, 40(6 SUPPL. 1), S192–S196. <https://doi.org/10.1016/j.jelectrocard.2007.05.035>
- Bird, S. D., Doevendans, P. A., van Rooijen, M. A., Brutel De La Riviere, A., Hassink, R. J., Passier, R., & Mummery, C. L. (2003). The human adult cardiomyocyte phenotype. *Cardiovascular Research*, 58(2), 423–434. [https://doi.org/10.1016/S0008-6363\(03\)00253-0](https://doi.org/10.1016/S0008-6363(03)00253-0)
- Blackman, B. E., Horner, K., Heidmann, J., Wang, D., Richter, W., Rich, T. C., & Conti, M. (2011). PDE4D and PDE4B function in distinct subcellular compartments in mouse embryonic fibroblasts. *Journal of Biological Chemistry*, 286(14), 12590–12601. <https://doi.org/10.1074/jbc.M110.203604>
- Börner, S., Schwede, F., Schlipp, A., Berisha, F., Calebiro, D., Lohse, M. J., & Nikolaev, V. O. (2011). FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nature Protocols*, 6(4), 427–438. <https://doi.org/10.1038/nprot.2010.198>

- Bosman, A., Sartiani, L., Spinelli, V., del Lungo, M., Stillitano, F., Nosi, D., Mugelli, A., Cerbai, E., & Jaconi, M. (2013). Molecular and functional evidence of HCN4 and caveolin-3 interaction during cardiomyocyte differentiation from human embryonic stem cells. *Stem Cells and Development*, 22(11), 1717–1727. <https://doi.org/10.1089/scd.2012.0247>
- Brandao, K. O., Tabel, V. A., Atsma, D. E., Mummery, C. L., & Davis, R. P. (2017). Human pluripotent stem cell models of cardiac disease: From mechanisms to therapies. In *DMM Disease Models and Mechanisms* (Vol. 10, Issue 9, pp. 1039–1059). Company of Biologists Ltd. <https://doi.org/10.1242/dmm.030320>
- Bray, M. A., Sheehy, S. P., & Parker, K. K. (2008a). Sarcomere alignment is regulated by myocyte shape. *Cell Motility and the Cytoskeleton*, 65(8), 641–651. <https://doi.org/10.1002/cm.20290>
- Brito-Martins, M., Harding, S. E., & Ali, N. N. (2008). β 1- and β 2-adrenoceptor responses in cardiomyocytes derived from human embryonic stem cells: Comparison with failing and non-failing adult human heart. *British Journal of Pharmacology*. <https://doi.org/10.1038/sj.bjp.0707619>
- Brodde, O. E. (1991). Beta 1- and beta 2-adrenoceptors in the human heart: properties, function, and alterations in chronic heart failure. *Pharmacological Reviews*, 43(2).
- Brook, W. H., Connell, S., Cannata, J., Maloney, J. E., & Walker, A. M. (1983). Ultrastructure of the myocardium during development from early fetal life to adult life in sheep. *Journal of Anatomy*, 137(Pt 4), 729.
- Brown, D. A. (2006). Lipid rafts, detergent-resistant membranes, and raft targeting signals. In *Physiology* (Vol. 21, Issue 6, pp. 430–439). American Physiological Society. <https://doi.org/10.1152/physiol.00032.2006>
- Brown, D. A., & Rose, J. K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell*, 68(3), 533–544. [https://doi.org/10.1016/0092-8674\(92\)90189-J](https://doi.org/10.1016/0092-8674(92)90189-J)
- Brown, L. A., & Harding, S. E. (1992). The effect of pertussis toxin on β -adrenoceptor responses in isolated cardiac myocytes from noradrenaline-treated guinea-pigs and patients with cardiac failure. *British Journal of Pharmacology*, 106(1), 115–122. <https://doi.org/10.1111/j.1476-5381.1992.tb14302.x>
- Bruck, H., Leineweber, K., Büscher, R., Ulrich, A., Radke, J., Insel, P. A., & Brodde, O. E. (2003). The Gln27Glu β 2-adrenoceptor polymorphism slows the onset of desensitization of cardiac functional responses in vivo. *Pharmacogenetics*, 13(2), 59–66. <https://doi.org/10.1097/00008571-200302000-00001>
- Buraei, Z., & Yang, J. (2013). Structure and function of the β subunit of voltage-gated Ca²⁺ channels. In *Biochimica et Biophysica Acta - Biomembranes* (Vol. 1828, Issue 7, pp. 1530–1540). NIH Public Access. <https://doi.org/10.1016/j.bbamem.2012.08.028>
- Burridge, P. W., Keller, G., Gold, J. D., & Wu, J. C. (2012). Production of de novo cardiomyocytes: Human pluripotent stem cell differentiation and direct reprogramming. In *Cell Stem Cell* (Vol. 10, Issue 1, pp. 16–28). Cell Press. <https://doi.org/10.1016/j.stem.2011.12.013>
- Burridge, P. W., Thompson, S., Millrod, M. A., Weinberg, S., Yuan, X., Peters, A., Mahairaki, V., Koliatsos, V. E., Tung, L., & Zambidis, E. T. (2011). A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS ONE*, 6(4). <https://doi.org/10.1371/journal.pone.0018293>

- Bursac, N., Parker, K. K., Iravanian, S., & Tung, L. (2002). Cardiomyocyte cultures with controlled macroscopic anisotropy: a model for functional electrophysiological studies of cardiac muscle. *Circulation Research*, 91(12). <https://doi.org/10.1161/01.RES.0000047530.88338.EB>
- Busija, A. R., Patel, H. H., & Insel, P. A. (2017). Caveolins and cavins in the trafficking, maturation, and degradation of caveolae: Implications for cell physiology. In *American Journal of Physiology - Cell Physiology* (Vol. 312, Issue 4, pp. C459–C477). American Physiological Society. <https://doi.org/10.1152/ajpcell.00355.2016>
- Cai, W., Zhang, J., de Lange, W. J., Gregorich, Z. R., Karp, H., Farrell, E. T., Mitchell, S. D., Tucholski, T., Lin, Z., Biermann, M., McIlwain, S. J., Ralphe, J. C., Kamp, T. J., & Ge, Y. (2019). An Unbiased Proteomics Method to Assess the Maturation of Human Pluripotent Stem Cell–Derived Cardiomyocytes. *Circulation Research*, 125(11), 936–953. <https://doi.org/10.1161/CIRCRESAHA.119.315305>
- Calaghan, S., Kozera, L., & White, E. (2008). Compartmentalisation of cAMP-dependent signalling by caveolae in the adult cardiac myocyte. *Journal of Molecular and Cellular Cardiology*, 45(1), 88–92. <https://doi.org/10.1016/j.yjmcc.2008.04.004>
- Calaghan, S., & White, E. (2006a). Caveolae modulate excitation-contraction coupling and β 2- adrenergic signalling in adult rat ventricular myocytes. *Cardiovascular Research*, 69(4), 816–824. <https://doi.org/10.1016/j.cardiores.2005.10.006>
- Calebiro, D., Rieken, F., Wagner, J., Sungkaworn, T., Zabel, U., Borzi, A., Cocucci, E., Zörn, A., & Lohse, M. J. (2013). Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proceedings of the National Academy of Sciences of the United States of America*, 110(2), 743–748. <https://doi.org/10.1073/pnas.1205798110>
- Camelliti, P., Gallagher, J. O., Kohl, P., & McCulloch, A. D. (2006). Micropatterned cell cultures on elastic membranes as an in vitro model of myocardium. *Nature Protocols*, 1(3), 1379–1391. <https://doi.org/10.1038/nprot.2006.203>
- Carlisle Michel, J. J., Dodge, K. L., Wong, W., Mayer, N. C., Langeberg, L. K., & Scott, J. D. (2004). PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. *Biochemical Journal*, 381(3), 587–592. <https://doi.org/10.1042/BJ20040846>
- Carlson, H. J., & Campbell, R. E. (2009). Genetically encoded FRET-based biosensors for multiparameter fluorescence imaging. In *Current Opinion in Biotechnology* (Vol. 20, Issue 1, pp. 19–27). Curr Opin Biotechnol. <https://doi.org/10.1016/j.copbio.2009.01.003>
- Carvajal-Vergara, X., Sevilla, A., Dsouza, S. L., Ang, Y. S., Schaniel, C., Lee, D. F., Yang, L., Kaplan, A. D., Adler, E. D., Rozov, R., Ge, Y., Cohen, N., Edelmann, L. J., Chang, B., Waghay, A., Su, J., Pardo, S., Lichtenbelt, K. D., Tartaglia, M., ... Lemischka, I. R. (2010). Patient-specific induced pluripotent stem-cell-derived models of LEOPARD syndrome. *Nature*, 465(7299), 808–812. <https://doi.org/10.1038/nature09005>
- CAV3 gene - Genetics Home Reference - NIH. (n.d.). Retrieved July 11, 2020, from <https://ghr.nlm.nih.gov/gene/CAV3>
- Chen, C., Termglinchan, V., & Karakikes, I. (2017). Concise Review: Mending a Broken Heart: The Evolution of Biological Therapeutics. *Stem Cells*, 35(5), 1131–1140. <https://doi.org/10.1002/stem.2602>
- Cheng, J. P. X., & Nichols, B. J. (2016). Caveolae: One Function or Many? In *Trends in Cell Biology* (Vol. 26, Issue 3, pp. 177–189). Elsevier Ltd. <https://doi.org/10.1016/j.tcb.2015.10.010>

- Chen-Izu, Y., Xiao, R. P., Izu, L. T., Cheng, H., Kuschel, M., Spurgeon, H., & Lakatta, E. G. (2000). G(i)-dependent localization of beta(2)-adrenergic receptor signaling to L-type Ca(2+) channels. *Biophysical Journal*, 79(5), 2547–2556. [https://doi.org/10.1016/S0006-3495\(00\)76495-2](https://doi.org/10.1016/S0006-3495(00)76495-2)
- Chini, B., & Parenti, M. (2004). G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? In *Journal of Molecular Endocrinology* (Vol. 32). <http://www.endocrinology.org>
- Chopra, A., Tabdanov, E., Patel, H., Janmey, P. A., & Kresh, J. Y. (2011). Cardiac myocyte remodeling mediated by N-cadherin-dependent mechanosensing. *American Journal of Physiology - Heart and Circulatory Physiology*, 300(4). <https://doi.org/10.1152/ajpheart.00515.2010>
- Chruscinski, A. J., Rohrer, D. K., Schauble, E., Desai, K. H., Bernstein, D., & Kobilka, B. K. (1999). Targeted disruption of the $\beta 2$ adrenergic receptor gene. *Journal of Biological Chemistry*, 274(24), 16694–16700. <https://doi.org/10.1074/jbc.274.24.16694>
- Cohen, A. W., Hnasko, R., Schubert, W., & Lisanti, M. P. (2004). Role of caveolae and caveolins in health and disease. In *Physiological Reviews* (Vol. 84, Issue 4, pp. 1341–1379). American Physiological Society. <https://doi.org/10.1152/physrev.00046.2003>
- Communal, Catherine, Singh, K., Sawyer, D. B., & Colucci, W. S. (1999). Opposing effects of $\beta 1$ - and $\beta 2$ -adrenergic receptors on cardiac myocyte apoptosis: Role of a pertussis toxin-sensitive G protein. *Circulation*, 100(22), 2210–2212. <https://doi.org/10.1161/01.CIR.100.22.2210>
- Conti, M., & Beavo, J. (2007). Biochemistry and Physiology of Cyclic Nucleotide Phosphodiesterases: Essential Components in Cyclic Nucleotide Signaling. *Annual Review of Biochemistry*, 76(1), 481–511. <https://doi.org/10.1146/annurev.biochem.76.060305.150444>
- Conti, M., Mika, D., & Richter, W. (2014). Cyclic AMP compartments and signaling specificity: Role of cyclic nucleotide phosphodiesterases. *The Journal of General Physiology*. <https://doi.org/10.1085/jgp.201311083>
- Correia, C., Koshkin, A., Duarte, P., Hu, D., Carido, M., Sebastião, M. J., Gomes-Alves, P., Elliott, D. A., Domian, I. J., Teixeira, A. P., Alves, P. M., & Serra, M. (2018). 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. *Biotechnology and Bioengineering*, 115(3), 630–644. <https://doi.org/10.1002/bit.26504>
- Correia, C., Koshkin, A., Duarte, P., Hu, D., Teixeira, A., Domian, I., Serra, M., & Alves, P. M. (2017). Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Scientific Reports*, 7(1), 1–17. <https://doi.org/10.1038/s41598-017-08713-4>
- Costa, K. D., Takayama, Y., McCulloch, A. D., & Covell, J. W. (1999). Laminar fiber architecture and three-dimensional systolic mechanics in canine ventricular myocardium. *American Journal of Physiology - Heart and Circulatory Physiology*, 276(2 45-2). <https://doi.org/10.1152/ajpheart.1999.276.2.h595>
- Cowan, C. A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J. P., Wang, S., Morton, C. C., McMahon, A. P., Powers, D., & Melton, D. A. (2004). Derivation of Embryonic Stem-Cell Lines from Human Blastocysts. *New England Journal of Medicine*, 350(13), 1353–1356. <https://doi.org/10.1056/NEJMSr040330>
- Cui, L., Johkura, K., Takei, S., Ogiwara, N., & Sasaki, K. (2007). Structural differentiation, proliferation, and association of human embryonic stem cell-derived cardiomyocytes in vitro and in their extracardiac tissues. *Journal of Structural Biology*, 158(3), 307–317. <https://doi.org/10.1016/j.jsb.2006.11.009>

- Daaka, Y., Luttrell, L. M., & Lefkowitz, R. J. (1997). Switching of the coupling of the β_2 -adrenergic receptor to different G proteins by protein kinase A. *Nature*, 390(6655), 88–91. <https://doi.org/10.1038/36362>
- Dai, D.-F., Danoviz, M. E., Wiczer, B., Laflamme, M. A., & Tian, R. (2017). Mitochondrial Maturation in Human Pluripotent Stem Cell Derived Cardiomyocytes. *Hindawi.Com*. <https://doi.org/10.1155/2017/5153625>
- Danielewicz, H. (2014). What the Genetic Background of Individuals with Asthma and Obesity Can Reveal: Is β_2 -Adrenergic Receptor Gene Polymorphism Important? *Pediatric Allergy, Immunology, and Pulmonology*, 27(3), 104–110. <https://doi.org/10.1089/ped.2014.0360>
- de Almeida, P. E., Ransohoff, J. D., Nahid, A., & Wu, J. C. (2013). Immunogenicity of Pluripotent Stem Cells and Their Derivatives. *Circulation Research*, 112(3), 549–561. <https://doi.org/10.1161/CIRCRESAHA.111.249243>
- de La Mata, A., Tajada, S., O'Dwyer, S., Matsumoto, C., Dixon, R. E., Hariharan, N., Moreno, C. M., & Santana, L. F. (2019). BIN1 Induces the Formation of T-Tubules and Adult-Like Ca^{2+} Release Units in Developing Cardiomyocytes. *Stem Cells*, 37(1), 54–64. <https://doi.org/10.1002/stem.2927>
- del Monte, F., Kaumann, A. J., Poole-Wilson, P. A., Wynne, D. G., Pepper, J., & Harding, S. E. (1993). Coexistence of functioning β_1 - and β_2 -adrenoceptors in single myocytes from human ventricle. *Circulation*, 88(3), 854–863. <https://doi.org/10.1161/01.CIR.88.3.854>
- Denning, C., Borgdorff, V., Crutchley, J., Firth, K. S. A., George, V., Kalra, S., Kondrashov, A., Hoang, M. D., Mosqueira, D., Patel, A., Prodanov, L., Rajamohan, D., Skarnes, W. C., Smith, J. G. W., & Young, L. E. (2016b). Cardiomyocytes from human pluripotent stem cells: From laboratory curiosity to industrial biomedical platform. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1863(7), 1728–1748. <https://doi.org/10.1016/j.bbamcr.2015.10.014>
- Desai, R. A., Rodriguez, N. M., & Chen, C. S. (2014). “Stamp-off” to micropattern sparse, multicomponent features. In *Methods in Cell Biology* (Vol. 119, pp. 3–16). Academic Press Inc. <https://doi.org/10.1016/B978-0-12-416742-1.00001-9>
- Dhamoon, A. S., & Jalife, J. (2005). The inward rectifier current (IK1) controls cardiac excitability and is involved in arrhythmogenesis. *Heart Rhythm*, 2(3), 316–324. <https://doi.org/10.1016/j.hrthm.2004.11.012>
- di Benedetto, G., Zoccarato, A., Lissandron, V., Terrin, A., Li, X., Houslay, M. D., Baillie, G. S., & Zaccolo, M. (2008). Protein kinase A type I and type II define distinct intracellular signaling compartments. *Circulation Research*, 103(8), 836–844. <https://doi.org/10.1161/CIRCRESAHA.108.174813>
- Dias, T. P., Pinto, S. N., Santos, J. I., Fernandes, T. G., Fernandes, F., Diogo, M. M., Prieto, M., & Cabral, J. M. S. (2018). Biophysical study of human induced Pluripotent Stem Cell-Derived cardiomyocyte structural maturation during long-term culture. *Biochemical and Biophysical Research Communications*, 499(3), 611–617. <https://doi.org/10.1016/j.bbrc.2018.03.198>
- Difrancesco, D. (2010). The role of the funny current in pacemaker activity. In *Circulation Research* (Vol. 106, Issue 3, pp. 434–446). Circ Res. <https://doi.org/10.1161/CIRCRESAHA.109.208041>
- Ding, B., Abe, J. I., Wei, H., Huang, Q., Walsh, R. A., Molina, C. A., Zhao, A., Sadoshima, J., Blaxall, B. C., Berk, B. C., & Yan, C. (2005). Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis:

Implication in heart failure. *Circulation*, 111(19), 2469–2476.
<https://doi.org/10.1161/01.CIR.0000165128.39715.87>

- Dishy, V., Sofowora, G. G., Xie, H. G., Kim, R. B., Byrne, D. W., Michael Stein, C., & Wood, A. J. J. (2001). The effect of common polymorphisms of the β 2-adrenergic receptor on agonist-mediated vascular desensitization. *New England Journal of Medicine*, 345(14), 1030–1035.
<https://doi.org/10.1056/NEJMoa010819>
- Dodge, K., Khouangsathiene, S., ... M. K.-T. E., & 2001, undefined. (n.d.). mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. *Embopress.Org*. Retrieved April 15, 2020, from <https://www.embopress.org/doi/full/10.1093/emboj/20.8.1921>
- Dodge-Kafka, K. L., Bauman, A., Mayer, N., Henson, E., Heredia, L., Ahn, J., McAvoy, T., Nairn, A. C., & Kapiloff, M. S. (2010). cAMP-stimulated protein phosphatase 2A activity associated with muscle a kinase-anchoring protein (mAKAP) signaling complexes inhibits the phosphorylation and activity of the cAMP-specific phosphodiesterase PDE4D3. *Journal of Biological Chemistry*, 285(15), 11078–11086. <https://doi.org/10.1074/jbc.M109.034868>
- Dodge-Kafka, K. L., Souhayer, J., Pare, G. C., Carlisle Michel, J. J., Langeberg, L. K., Kapiloff, M. S., & Scott, J. D. (2005). The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. *Nature*, 437(7058), 574–578. <https://doi.org/10.1038/nature03966>
- Dolnikov, K., Shilkrut, M., Zeevi-Levin, N., Gerecht-Nir, S., Amit, M., Danon, A., Itskovitz-Eldor, J., & Binah, O. (2006). Functional Properties of Human Embryonic Stem Cell-Derived Cardiomyocytes: Intracellular Ca²⁺ Handling and the Role of Sarcoplasmic Reticulum in the Contraction. *Stem Cells*, 24(2), 236–245. <https://doi.org/10.1634/stemcells.2005-0036>
- Dooley, D. J., Bittiger, H., & Reymann, N. C. (1986). CGP 20712 A: a useful tool for quantitating β 1- and β 2-adrenoceptors. *European Journal of Pharmacology*, 130(1–2), 137–139.
[https://doi.org/10.1016/0014-2999\(86\)90193-7](https://doi.org/10.1016/0014-2999(86)90193-7)
- Duval, K., Grover, H., Han, L. H., Mou, Y., Pegoraro, A. F., Fredberg, J., & Chen, Z. (2017). Modeling physiological events in 2D vs. 3D cell culture. In *Physiology* (Vol. 32, Issue 4, pp. 266–277). American Physiological Society. <https://doi.org/10.1152/physiol.00036.2016>
- Egorov, Y. v., Lang, D., Tyan, L., Turner, D., Lim, E., Piro, Z. D., Hernandez, J. J., Lodin, R., Wang, R., Schmuck, E. G., Raval, A. N., Ralphe, C. J., Kamp, T. J., Rosenshtraukh, L. v., & Glukhov, A. v. (2019). Caveolae-Mediated Activation of Mechanosensitive Chloride Channels in Pulmonary Veins Triggers Atrial Arrhythmogenesis. *Journal of the American Heart Association*, 8(20).
<https://doi.org/10.1161/JAHA.119.012748>
- El, P., Scott Hayes, J., Brunton, L. L., & Mayer, S. E. (1980). Selective Activation of Particulate CAMP-dependent Protein Kinase by. In *THE JOURNAL OF BIOLOGICAL CHEMISTRY* (Vol. 255, Issue 11). <http://www.jbc.org/content/255/11/5113.short>
- Engelhardt, S., Böhm, M., Erdmann, E., & Lohse, M. J. (1996). Analysis of beta-adrenergic receptor mRNA levels in human ventricular biopsy specimens by quantitative polymerase chain reactions: Progressive reduction of beta1-adrenergic receptor mRNA in heart failure. *Journal of the American College of Cardiology*, 27(1), 146–154. [https://doi.org/10.1016/0735-1097\(95\)00425-4](https://doi.org/10.1016/0735-1097(95)00425-4)
- Engler, A. J., Carag-Krieger, C., Johnson, C. P., Raab, M., Tang, H. Y., Speicher, D. W., Sanger, J. W., Sanger, J. M., & Discher, D. E. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like

elasticity: Scar-like rigidity inhibits beating. *Journal of Cell Science*, 121(22), 3794–3802.
<https://doi.org/10.1242/jcs.029678>

Epstein, P. M. (2017). Different phosphodiesterases (PDEs) regulate distinct phosphoproteomes during cAMP signaling. In *Proceedings of the National Academy of Sciences of the United States of America* (Vol. 114, Issue 30, pp. 7741–7743). National Academy of Sciences.
<https://doi.org/10.1073/pnas.1709073114>

Eschenhagen, T. (2013). PDE4 in the human heart - major player or little helper? *British Journal of Pharmacology*, 169(3), 524–527. <https://doi.org/10.1111/bph.12168>

Evans, W. E., & McLeod, H. L. (2003). Pharmacogenomics - Drug disposition, drug targets, and side effects. In A. J. J. Wood (Ed.), *New England Journal of Medicine* (Vol. 348, Issue 6, pp. 538–549). Massachusetts Medical Society . <https://doi.org/10.1056/NEJMra020526>

Feinberg, A. W., Alford, P. W., Jin, H., Ripplinger, C. M., Werdich, A. A., Sheehy, S. P., Grosberg, A., & Parker, K. K. (2012). Controlling the contractile strength of engineered cardiac muscle by hierarchal tissue architecture. *Biomaterials*, 33(23), 5732–5741.
<https://doi.org/10.1016/j.biomaterials.2012.04.043>

Feric, N. T., & Radisic, M. (2016). Maturing human pluripotent stem cell-derived cardiomyocytes in human engineered cardiac tissues. In *Advanced Drug Delivery Reviews* (Vol. 96, pp. 110–134). Elsevier B.V.
<https://doi.org/10.1016/j.addr.2015.04.019>

Ferreira-Santos, L., Martinez, D. G., Nicolau, J. C., Moreira, H. G., Alves, M. J., Pereira, A. C., Trombetta, I. C., Negrão, C. E., & Rondon, M. U. P. B. (2017). Neurovascular control during exercise in acute coronary syndrome patients with Gln27Glu polymorphism of β 2-adrenergic receptor. *PLoS ONE*, 12(2). <https://doi.org/10.1371/journal.pone.0173061>

Figtree, G. A., Bubbs, K. J., Tang, O., Kizana, E., & Gentile, C. (2017). Vascularized Cardiac Spheroids as Novel 3D in vitro Models to Study Cardiac Fibrosis. *Cells Tissues Organs*, 204(3–4), 191–198.
<https://doi.org/10.1159/000477436>

Fischmeister, R., Castro, L., Abi-Gerges, A., Rochais, F., & Vandecasteele, G. (2005). Species- and tissue-dependent effects of NO and cyclic GMP on cardiac ion channels. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, 142(2), 136–143.
<https://doi.org/10.1016/j.cbpb.2005.04.012>

Fischmeister, R., Castro, L. R. V., Abi-Gerges, A., Rochais, F., Jurevičius, J., Leroy, J., & Vandecasteele, G. (2006). Compartmentation of cyclic nucleotide signaling in the heart: The role of cyclic nucleotide phosphodiesterases. In *Circulation Research* (Vol. 99, Issue 8, pp. 816–828). Lippincott Williams and Wilkins. <https://doi.org/10.1161/01.RES.0000246118.98832.04>

Foerster, K., Groner, F., Matthes, J., Koch, W. J., Birnbaumer, L., & Herzig, S. (2003). Cardioprotection specific for the G protein G i2 in chronic adrenergic signaling through α 2-adrenoceptors. In *National Acad Sciences*. www.pnas.org/cgi/doi/10.1073/pnas.1936026100

Fox, I. J., Daley, G. Q., Goldman, S. A., Huard, J., Kamp, T. J., & Trucco, M. (2014). Use of differentiated pluripotent stem cells in replacement therapy for treating disease. In *Science* (Vol. 345, Issue 6199, p. 1247391). American Association for the Advancement of Science.
<https://doi.org/10.1126/science.1247391>

- Francis, S. H., Blount, M. A., & Corbin, J. D. (2011). Mammalian cyclic nucleotide phosphodiesterases: Molecular mechanisms and physiological functions. *Physiological Reviews*, 91(2), 651–690. <https://doi.org/10.1152/physrev.00030.2010>
- Gahbauer, S., & Böckmann, R. A. (2016). Membrane-mediated oligomerization of G protein coupled receptors and its implications for GPCR function. In *Frontiers in Physiology* (Vol. 7, Issue OCT, p. 494). Frontiers Media S.A. <https://doi.org/10.3389/fphys.2016.00494>
- Geisse, N. A., Sheehy, S. P., & Parker, K. K. (2009). Control of myocyte remodeling in vitro with engineered substrates. *In Vitro Cellular & Developmental Biology. Animal*, 45(7), 343–350. <https://doi.org/10.1007/s11626-009-9182-9>
- Germanguz, I., Sedan, O., Zeevi-Levin, N., Shtrichman, R., Barak, E., Ziskind, A., Eliyahu, S., Meiry, G., Amit, M., Itskovitz-Eldor, J., & Binah, O. (2011). Molecular characterization and functional properties of cardiomyocytes derived from human inducible pluripotent stem cells. *Journal of Cellular and Molecular Medicine*, 15(1), 38–51. <https://doi.org/10.1111/j.1582-4934.2009.00996.x>
- Ghadri, J., Wittstein, I., ... A. P.-E. heart, & 2018, undefined. (n.d.). International expert consensus document on Takotsubo syndrome (part I): clinical characteristics, diagnostic criteria, and pathophysiology. *Academic.Oup.Com*. Retrieved January 24, 2021, from <https://academic.oup.com/eurheartj/article-abstract/39/22/2032/5025412>
- Gherghiceanu, M., Barad, L., Novak, A., Reiter, I., Itskovitz-Eldor, J., Binah, O., & Popescu, L. M. (2011a). Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: Comparative ultrastructure. *Journal of Cellular and Molecular Medicine*, 15(11), 2539–2551. <https://doi.org/10.1111/j.1582-4934.2011.01417.x>
- Giacomelli, E., Mummery, C. L., & Bellin, M. (2017). Human heart disease: lessons from human pluripotent stem cell-derived cardiomyocytes. In *Cellular and Molecular Life Sciences* (Vol. 74, Issue 20, pp. 3711–3739). Birkhauser Verlag AG. <https://doi.org/10.1007/s00018-017-2546-5>
- Gilman, A. G. (1984). G proteins and dual control of adenylate cyclase. In *Cell* (Vol. 36, Issue 3, pp. 577–579). Elsevier. [https://doi.org/10.1016/0092-8674\(84\)90336-2](https://doi.org/10.1016/0092-8674(84)90336-2)
- Gintant, G., Burridge, P., Gepstein, L., Harding, S., Herron, T., Hong, C., Jalife, J., & Wu, J. C. (2019). Use of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in Preclinical Cancer Drug Cardiotoxicity Testing: A Scientific Statement From the American Heart Association. *Circulation Research*, 125(10), e75–e92. <https://doi.org/10.1161/RES.0000000000000291>
- Gnecchi, M., Zhang, Z., Ni, A., & Dzau, V. J. (2008). Paracrine mechanisms in adult stem cell signaling and therapy. In *Circulation Research* (Vol. 103, Issue 11, pp. 1204–1219). Circ Res. <https://doi.org/10.1161/CIRCRESAHA.108.176826>
- Göktepe, S., Abilez, O. J., Parker, K. K., & Kuhl, E. (2010). A multiscale model for eccentric and concentric cardiac growth through sarcomerogenesis. *Journal of Theoretical Biology*, 265(3), 433–442. <https://doi.org/10.1016/j.jtbi.2010.04.023>
- Golob, M., Moss, R. L., & Chesler, N. C. (2014). Cardiac Tissue Structure, Properties, and Performance: A Materials Science Perspective. In *Annals of Biomedical Engineering* (Vol. 42, Issue 10, pp. 2003–2013). Kluwer Academic Publishers. <https://doi.org/10.1007/s10439-014-1071-z>

- Gorelik, J., Yang, L. Q., Zhang, Y., Lab, M., Korchev, Y., & Harding, S. E. (2006). A novel Z-groove index characterizing myocardial surface structure. *Cardiovascular Research*, 72(3), 422–429. <https://doi.org/10.1016/j.cardiores.2006.09.009>
- Gough, N. R. (2011). Focus issue: Cracking the G protein-coupled receptor code. In *Science Signaling* (Vol. 4, Issue 185, pp. eg7–eg7). American Association for the Advancement of Science. <https://doi.org/10.1126/scisignal.2002388>
- Goversen, B., van der Heyden, M. A. G., van Veen, T. A. B., & de Boer, T. P. (2018). The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on IK1. In *Pharmacology and Therapeutics* (Vol. 183, pp. 127–136). Elsevier Inc. <https://doi.org/10.1016/j.pharmthera.2017.10.001>
- Green, S. A., Turki, J., Bejarano, P., Hall, I. P., & Liggett, S. B. (1995). Influence of beta 2-adrenergic receptor genotypes on signal transduction in human airway smooth muscle cells. *American Journal of Respiratory Cell and Molecular Biology*, 13(1), 25–33. <https://doi.org/10.1165/ajrcmb.13.1.7598936>
- Green, Stuart A., Turki, J., Liggett, S. B., & Innis, M. (1994). Amino-Terminal Polymorphisms of the Human β 2-Adrenergic Receptor Impart Distinct Agonist-Promoted Regulatory Properties. *Biochemistry*, 33(32), 9414–9419. <https://doi.org/10.1021/bi00198a006>
- Gregorio, C. C., & Antin, P. B. (2000). To the heart of myofibril assembly. *Trends in Cell Biology*, 10(9), 355–362. [https://doi.org/10.1016/S0962-8924\(00\)01793-1](https://doi.org/10.1016/S0962-8924(00)01793-1)
- Gridelli, B., & Remuzzi, G. (2000). Strategies for making more organs available for transplantation. In *New England Journal of Medicine* (Vol. 343, Issue 6, pp. 404–410). Massachusetts Medical Society . <https://doi.org/10.1056/NEJM200008103430606>
- Grimaldi, V., Mancini, F. P., Casamassimi, A., Al-Omran, M., Zullo, A., Infante, T., & Napoli, C. (2013). Potential benefits of cell therapy in coronary heart disease. In *Journal of Cardiology* (Vol. 62, Issue 5, pp. 267–276). Elsevier. <https://doi.org/10.1016/j.jjcc.2013.05.017>
- Guellich, A., Mehel, H., & Fischmeister, R. (2014). Cyclic AMP synthesis and hydrolysis in the normal and failing heart. In *Pflugers Archiv European Journal of Physiology* (Vol. 466, Issue 6, pp. 1163–1175). Springer Verlag. <https://doi.org/10.1007/s00424-014-1515-1>
- Guven, S., Chen, P., Inci, F., Tasoglu, S., Erkmen, B., & Demirci, U. (2015). Multiscale assembly for tissue engineering and regenerative medicine. In *Trends in Biotechnology* (Vol. 33, Issue 5, pp. 269–279). Elsevier Ltd. <https://doi.org/10.1016/j.tibtech.2015.02.003>
- Habib, M., Caspi, O., & Gepstein, L. (2008). Human embryonic stem cells for cardiomyogenesis. In *Journal of Molecular and Cellular Cardiology* (Vol. 45, Issue 4, pp. 462–474). Academic Press. <https://doi.org/10.1016/j.yjmcc.2008.08.008>
- Hambleton, R., Krall, J., Tikishvili, E., Honeggar, M., Ahmad, F., Manganiello, V. C., & Movsesian, M. A. (2005a). Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP hydrolytic activity in subcellular fractions of human myocardium. *Journal of Biological Chemistry*, 280(47), 39168–39174. <https://doi.org/10.1074/jbc.M506760200>
- Hambleton, R., Krall, J., Tikishvili, E., Honeggar, M., Ahmad, F., Manganiello, V. C., & Movsesian, M. A. (2005b). Isoforms of cyclic nucleotide phosphodiesterase PDE3 and their contribution to cAMP

- hydrolytic activity in subcellular fractions of human myocardium. *Journal of Biological Chemistry*, 280(47), 39168–39174. <https://doi.org/10.1074/jbc.M506760200>
- Hansma, P. K., Drake, B., Marti, O., Gould, S. A. C., & Prater, C. B. (1989). The scanning ion-conductance microscope. *Science*, 243(4891), 641–643. <https://doi.org/10.1126/science.2464851>
- Harvey, R. D., & Calaghan, S. C. (2012). Caveolae create local signalling domains through their distinct protein content, lipid profile and morphology. In *Journal of Molecular and Cellular Cardiology* (Vol. 52, Issue 2, pp. 366–375). <https://doi.org/10.1016/j.yjmcc.2011.07.007>
- Head, B. P., Patel, H. H., Roth, D. M., Murray, F., Swaney, J. S., Niesman, I. R., Farquhar, M. G., & Insel, P. A. (2006). Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. *Journal of Biological Chemistry*, 281(36), 26391–26399. <https://doi.org/10.1074/jbc.M602577200>
- Head, B. P., Pateli, H. H., Roth, D. M., Lai, N. C., Niesman, I. R., Farquhar, M. G., & Insel, P. A. (2005). G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *Journal of Biological Chemistry*, 280(35), 31036–31044. <https://doi.org/10.1074/jbc.M502540200>
- Henderson, C. A., Gomez, C. G., Novak, S. M., Mi-Mi, L., & Gregorio, C. C. (2017). Overview of the Muscle Cytoskeleton. *Comprehensive Physiology*, 7(3), 891. <https://doi.org/10.1002/CPHY.C160033>
- Heubach, J. F., Ravens, U., & Kaumann, A. J. (2004). Epinephrine Activates Both Gs and Gi Pathways, but Norepinephrine Activates Only the Gs Pathway through Human β -Adrenoceptors Overexpressed in Mouse Heart. *Molecular Pharmacology*, 65(5), 1313–1322. <https://doi.org/10.1124/mol.65.5.1313>
- Heubach, J., Ravens, U., pharmacology, A. K.-M., & 2004, undefined. (n.d.). Epinephrine activates both Gs and Gi pathways, but norepinephrine activates only the Gs pathway through human β 2-adrenoceptors overexpressed in mouse heart. *ASPET*. Retrieved April 18, 2020, from <http://molpharm.aspetjournals.org/content/65/5/1313.short>
- Hochedlinger, K., & Plath, K. (2009). Epigenetic reprogramming and induced pluripotency. In *Development* (Vol. 136, Issue 4, pp. 509–523). The Company of Biologists Ltd. <https://doi.org/10.1242/dev.020867>
- Hockemeyer, D., & Jaenisch, R. (2016). Induced pluripotent stem cells meet genome editing. In *Cell Stem Cell* (Vol. 18, Issue 5, pp. 573–586). Cell Press. <https://doi.org/10.1016/j.stem.2016.04.013>
- Hoekstra, M., Mummery, C. L., Wilde, A. A. M., Bezzina, C. R., & Verkerk, A. O. (2012). Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias. *Frontiers in Physiology*, 3, 346. <https://doi.org/10.3389/fphys.2012.00346>
- Hoover, D. B., Ganote, C. E., Ferguson, S. M., Blakely, R. D., & Parsons, R. L. (2004). Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. *Cardiovascular Research*, 62(1), 112–121. <https://doi.org/10.1016/j.cardiores.2004.01.012>
- Horinouchi, T., Tanaka, Y., & Koike, K. (2003). Function of β 1-adrenoceptors and mRNA expression of β 1- and β 2-adrenoceptors in guinea-pig esophagus. *European Journal of Pharmacology*, 473(1), 79–82. [https://doi.org/10.1016/S0014-2999\(03\)01943-5](https://doi.org/10.1016/S0014-2999(03)01943-5)
- Huebsch, N., Loskill, P., Deveshwar, N., Ian Spencer, C., Judge, L. M., Mandegar, M. A., Fox, C. B., Mohamed, T. M., Ma, Z., Mathur, A., Sheehan, A. M., Truong, A., Saxton, M., Yoo, J., Srivastava, D., Desai, T. A., So, P.-L., Healy, K. E., & Conklin, B. R. (2016). Miniaturized iPS-Cell-Derived Cardiac

Muscles for Physiologically Relevant Drug Response Analyses OPEN. *Nature Publishing Group*.
<https://doi.org/10.1038/srep24726>

Huethorst, E., Hortigon, M., Zamora-Rodriguez, V., Reynolds, P. M., Burton, F., Smith, G., & Gadegaard, N. (2016). Enhanced Human-Induced Pluripotent Stem Cell Derived Cardiomyocyte Maturation Using a Dual Microgradient Substrate. *ACS Biomaterials Science and Engineering*, 2(12), 2231–2239.
<https://doi.org/10.1021/acsbiomaterials.6b00426>

Huynh, T. v., Chen, F., Wetzel, G. T., Friedman, W. F., & Klitzner, T. S. (1992). Developmental changes in membrane Ca²⁺ and K⁺ currents in fetal, neonatal, and adult rabbit ventricular myocytes. *Circulation Research*, 70(3), 508–515. <https://doi.org/10.1161/01.res.70.3.508>

Hwang, P. M., & Sykes, B. D. (2015). Targeting the sarcomere to correct muscle function. In *Nature Reviews Drug Discovery* (Vol. 14, Issue 5, pp. 313–328). Nature Publishing Group.
<https://doi.org/10.1038/nrd4554>

Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell*, 142(3), 375–386. <https://doi.org/10.1016/j.cell.2010.07.002>

Ieda, M., Kanazawa, H., Kimura, K., Hattori, F., Ieda, Y., Taniguchi, M., Lee, J. K., Matsumura, K., Tomita, Y., Miyoshi, S., Shimoda, K., Makino, S., Sano, M., Kodama, I., Ogawa, S., & Fukuda, K. (2007). Sema3a maintains normal heart rhythm through sympathetic innervation patterning. *Nature Medicine*, 13(5), 604–612. <https://doi.org/10.1038/nm1570>

Itzhaki, I., Rapoport, S., Huber, I., Mizrahi, I., Zwi-Dantsis, L., Arbel, G., Schiller, J., & Gepstein, L. (2011). Calcium handling in human induced pluripotent stem cell derived cardiomyocytes. *PLoS ONE*, 6(4).
<https://doi.org/10.1371/journal.pone.0018037>

Iwamoto, Y., Ohishi, M., Yuan, M., Tatara, Y., Kato, N., Takeya, Y., Onishi, M., Maekawa, Y., Kamide, K., & Rakugi, H. (2011). B-Adrenergic receptor gene polymorphism is a genetic risk factor for cardiovascular disease: A cohort study with hypertensive patients. *Hypertension Research*, 34(5), 573–577. <https://doi.org/10.1038/hr.2010.281>

Jacobson, K., Mouritsen, O. G., & Anderson, R. G. W. (2007). Lipid rafts: At a crossroad between cell biology and physics. In *Nature Cell Biology* (Vol. 9, Issue 1, pp. 7–14). Nature Publishing Group.
<https://doi.org/10.1038/ncb0107-7>

James, T., & TN, J. (1982). *Neural control of the heart in health and disease*. <https://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=PASCAL82X0378500>

Johnson, J. A., & Terra, S. G. (2002). β -adrenergic receptor polymorphisms: Cardiovascular disease associations and pharmacogenetics. In *Pharmaceutical Research* (Vol. 19, Issue 12, pp. 1779–1787). Springer. <https://doi.org/10.1023/A:1021477021102>

Jung, G., Fajardo, G., Ribeiro, A. J. S., Kooiker, K. B., Coronado, M., Zhao, M., Hu, D. Q., Reddy, S., Kodo, K., Sriram, K., Insel, P. A., Wu, J. C., Pruitt, B. L., & Bernstein, D. (2016). Time-dependent evolution of functional vs. remodeling signaling in induced pluripotent stem cell-derived cardiomyocytes and induced maturation with biomechanical stimulation. *FASEB Journal*. <https://doi.org/10.1096/fj.15-280982>

- Jurevičius, J., & Fischmeister, R. (1996). cAMP compartmentation is responsible for a local activation of cardiac Ca²⁺ channels by β -adrenergic agonists. *Proceedings of the National Academy of Sciences of the United States of America*, 93(1), 295–299. <https://doi.org/10.1073/pnas.93.1.295>
- Kaitin, K. I. (2010). Deconstructing the drug development process: The new face of innovation. In *Clinical Pharmacology and Therapeutics* (Vol. 87, Issue 3, pp. 356–361). Nature Publishing Group. <https://doi.org/10.1038/clpt.2009.293>
- Kaji, H., Takii, Y., Nishizawa, M., & Matsue, T. (2003). Pharmacological characterization of micropatterned cardiac myocytes. *Biomaterials*, 24(23), 4239–4244. [https://doi.org/10.1016/S0142-9612\(03\)00275-8](https://doi.org/10.1016/S0142-9612(03)00275-8)
- Kaji, H., Takoh, K., Nishizawa, M., & Matsue, T. (2003). Intracellular Ca²⁺ imaging for micropatterned cardiac myocytes. *Biotechnology and Bioengineering*, 81(6), 748–751. <https://doi.org/10.1002/bit.10521>
- Kamakura, T., Makiyama, T., Sasaki, K., Yoshida, Y., Wuriyanghai, Y., Chen, J., Hattori, T., Ohno, S., Kita, T., Horie, M., Yamanaka, S., & Kimura, T. (2013). Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture. *Circulation Journal*, 77(5), 1307–1314. <https://doi.org/10.1253/circj.CJ-12-0987>
- Kane, C., Couch, L., & Terracciano, C. M. N. (2015). Excitation-contraction coupling of human induced pluripotent stem cell-derived cardiomyocytes. In *Frontiers in Cell and Developmental Biology* (Vol. 3, Issue SEP). Frontiers Media S.A. <https://doi.org/10.3389/fcell.2015.00059>
- Karakikes, I., Ameen, M., Termglinchan, V., & Wu, J. C. (2015a). Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes: Insights into Molecular, Cellular, and Functional Phenotypes. In *Circulation Research* (Vol. 117, Issue 1, pp. 80–88). Lippincott Williams and Wilkins. <https://doi.org/10.1161/CIRCRESAHA.117.305365>
- Karakikes, I., Ameen, M., Termglinchan, V., & Wu, J. C. (2015b). Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes: Insights into Molecular, Cellular, and Functional Phenotypes. In *Circulation Research* (Vol. 117, Issue 1, pp. 80–88). Lippincott Williams and Wilkins. <https://doi.org/10.1161/CIRCRESAHA.117.305365>
- Karbassi, E., Fenix, A., Marchiano, S., Muraoka, N., Nakamura, K., Yang, X., & Murry, C. E. (2020). Cardiomyocyte maturation: advances in knowledge and implications for regenerative medicine. In *Nature Reviews Cardiology* (pp. 1–19). Nature Research. <https://doi.org/10.1038/s41569-019-0331-x>
- Kaumann, A., Bartel, S., Molenaar, P., Sanders, L., Burrell, K., Vetter, D., Hempel, P., Karczewski, P., & Krause, E. G. (1999a). Activation of β 2-adrenergic receptors hastens relaxation and mediates phosphorylation of phospholamban, troponin I, and C-protein in ventricular myocardium from patients with terminal heart failure. *Circulation*, 99(1), 65–72. <https://doi.org/10.1161/01.CIR.99.1.65>
- Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., & Gepstein, L. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *Journal of Clinical Investigation*, 108(3), 407–414. <https://doi.org/10.1172/jci12131>
- Kelm, J. M., Ehler, E., Nielsen, L. K., Schlatter, S., Perriard, J. C., & Fussenegger, M. (2004). Design of Artificial Myocardial Microtissues. *Tissue Engineering*, 10(1–2), 201–214. <https://doi.org/10.1089/107632704322791853>

- Khan, J. M., Lyon, A. R., & Harding, S. E. (2013). The case for induced pluripotent stem cell-derived cardiomyocytes in pharmacological screening. *British Journal of Pharmacology*, 169(2), 304–317. <https://doi.org/10.1111/j.1476-5381.2012.02118.x>
- Khan, M., Mohsin, S., Avitabile, D., Siddiqi, S., Nguyen, J., Wallach, K., Quijada, P., McGregor, M., Gude, N., Alvarez, R., Tilley, D. G., Koch, W. J., & Sussman, M. A. (2013). β -Adrenergic regulation of cardiac progenitor cell death versus survival and proliferation. *Circulation Research*, 112(3), 476–486. <https://doi.org/10.1161/CIRCRESAHA.112.280735>
- Kijlstra, J. D., Hu, D., van der Meer, P., & Domian, I. J. (2017). Single-Cell Functional Analysis of Stem-Cell Derived Cardiomyocytes on Micropatterned Flexible Substrates. *Current Protocols in Stem Cell Biology*, 43(1), 1F.20.1–1F.20.9. <https://doi.org/10.1002/cpsc.40>
- Kim, D. H., Lipke, E. A., Kim, P., Cheong, R., Thompson, S., Delannoy, M., Suh, K. Y., Tung, L., & Levchenko, A. (2010). Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. *Proceedings of the National Academy of Sciences of the United States of America*, 107(2), 565–570. <https://doi.org/10.1073/pnas.0906504107>
- Kim, G. E., & Kass, D. A. (2017). Cardiac phosphodiesterases and their modulation for treating heart disease. In *Handbook of Experimental Pharmacology* (Vol. 243, pp. 249–269). Springer New York LLC. https://doi.org/10.1007/164_2016_82
- Kim, H., dirk, Kim, D. joong, Lee, I. jae, Rah, B. jin, Sawa, Y., & Schaper, J. (1992). Human fetal heart development after mid-term: Morphometry and ultrastructural study. *Journal of Molecular and Cellular Cardiology*, 24(9), 949–965. [https://doi.org/10.1016/0022-2828\(92\)91862-Y](https://doi.org/10.1016/0022-2828(92)91862-Y)
- Kimura, K., Ieda, M., & Fukuda, K. (2012). Development, maturation, and transdifferentiation of cardiac sympathetic nerves. *Circulation Research*, 110(2), 325–336. <https://doi.org/10.1161/CIRCRESAHA.111.257253>
- Klarenbeek, J. B., Goedhart, J., Hink, M. A., Gadella, T. W. J., & Jalink, K. (2011). A mTurquoise-Based cAMP Sensor for Both FLIM and Ratiometric Read-Out Has Improved Dynamic Range. *PLoS ONE*, 6(4), e19170. <https://doi.org/10.1371/journal.pone.0019170>
- Klein, I., & Ojamaa, K. (2001). Thyroid hormone and the cardiovascular system. In *New England Journal of Medicine* (Vol. 344, Issue 7, pp. 501–509). N Engl J Med. <https://doi.org/10.1056/NEJM200102153440707>
- Knight, W. E., & Yan, C. (2012). Cardiac cyclic nucleotide phosphodiesterases: Function, regulation, and therapeutic prospects. In *Hormone and Metabolic Research* (Vol. 44, Issue 10, pp. 766–775). NIH Public Access. <https://doi.org/10.1055/s-0032-1321870>
- Knollmann, B. C. (2013). Induced pluripotent stem cell-derived cardiomyocytes: boutique science or valuable arrhythmia model? *Circulation Research*, 112(6), 969–976; discussion 976. <https://doi.org/10.1161/CIRCRESAHA.112.300567>
- Koga, A., Oka, N., Kikuchi, T., Miyazaki, H., Kato, S., & Imaizumi, T. (2003). Adenovirus-mediated overexpression of caveolin-3 inhibits rat cardiomyocyte hypertrophy. *Hypertension*, 42(2), 213–219. <https://doi.org/10.1161/01.HYP.0000082926.08268.5D>
- Koivumäki, J. T., Naumenko, N., Tuomainen, T., Takalo, J., Oksanen, M., Puttonen, K. A., Lehtonen, Š., Kuusisto, J., Laakso, M., Koistinaho, J., & Tavi, P. (2018). Structural immaturity of human iPSC-derived

cardiomyocytes: In silico investigation of effects on function and disease modeling. *Frontiers in Physiology*, 9(FEB). <https://doi.org/10.3389/fphys.2018.00080>

Kondrashov, A., Hoang, M. D., Smith, J. G. W., Bhagwan, J. R., Duncan, G., Mosqueira, D., Munoz, M. B., Vo, N. T. N., & Denning, C. (2018). Simplified Footprint-Free Cas9/CRISPR Editing of Cardiac-Associated Genes in Human Pluripotent Stem Cells. *Stem Cells and Development*, 27(6), 391–404. <https://doi.org/10.1089/scd.2017.0268>

Korchev, Y. E., Lindsay Bashford, C., Milovanovic, M., Vodyanoy, I., & Lab, M. J. (1997). Scanning Ion Conductance Microscopy of Living Cells. In *Biophysical Journal* (Vol. 73).

Koryakina, Y., Jones, S. M., Cornett, L. E., Seely, K., Brents, L., Prather, P. L., Kofman, A., & Kurten, R. C. (2012). Effects of the β -agonist, isoprenaline, on the down-regulation, functional responsiveness and trafficking of β_2 -adrenergic receptors with N-terminal polymorphisms. *Cell Biology International*, 36(12), 1171–1183. <https://doi.org/10.1042/cbi20120134>

Kostic, M. M., Erdogan, S., Rena, G., Borchert, G., Hoch, B., Bartel, S., Scotland, G., Huston, E., Houslay, M. D., & Krause, E. G. (1997). Altered expression of PDE1 and PDE4 cyclic nucleotide phosphodiesterase isoforms in 7-oxo-prostacyclin-preconditioned rat heart. *Journal of Molecular and Cellular Cardiology*, 29(11), 3135–3146. <https://doi.org/10.1006/jmcc.1997.0544>

Koudstaal, S., Jansen of Lorkeers, S. J., Gaetani, R., Ghoe, J. M. I. H., van Slochteren, F. J., Sluijter, J. P. G., Doevendans, P. A., Ellison, G. M., & Chamuleau, S. A. J. (2013). Concise Review: Heart Regeneration and the Role of Cardiac Stem Cells. *STEM CELLS Translational Medicine*, 2(6), 434–443. <https://doi.org/10.5966/sctm.2013-0001>

Krüger, M., Sachse, C., Zimmermann, W. H., Eschenhagen, T., Klede, S., & Linke, W. A. (2008). Thyroid hormone regulates developmental titin isoform transitions via the phosphatidylinositol-3-kinase/Akt pathway. *Circulation Research*, 102(4), 439–447. <https://doi.org/10.1161/CIRCRESAHA.107.162719>

Kuo, P. L., Lee, H., Bray, M. A., Geisse, N. A., Huang, Y. T., Adams, W. J., Sheehy, S. P., & Parker, K. K. (2012a). Myocyte shape regulates lateral registry of sarcomeres and contractility. *American Journal of Pathology*, 181(6), 2030–2037. <https://doi.org/10.1016/j.ajpath.2012.08.045>

Kuo, P. L., Lee, H., Bray, M. A., Geisse, N. A., Huang, Y. T., Adams, W. J., Sheehy, S. P., & Parker, K. K. (2012b). Myocyte shape regulates lateral registry of sarcomeres and contractility. *American Journal of Pathology*, 181(6), 2030–2037. <https://doi.org/10.1016/j.ajpath.2012.08.045>

Kuschel, M., Zhou, Y.-Y., Spurgeon, H. A., Bartel, S., Karczewski, P., Zhang, S.-J., Krause, E.-G., Lakatta, E. G., & Xiao, R.-P. (1999). β_2 -Adrenergic cAMP Signaling Is Uncoupled From Phosphorylation of Cytoplasmic Proteins in Canine Heart. *Circulation*, 99(18), 2458–2465. <https://doi.org/10.1161/01.CIR.99.18.2458>

Laflamme, M. A., Chen, K. Y., Naumova, A. v., Muskheli, V., Fugate, J. A., Dupras, S. K., Reinecke, H., Xu, C., Hassanipour, M., Police, S., O'Sullivan, C., Collins, L., Chen, Y., Minami, E., Gill, E. A., Ueno, S., Yuan, C., Gold, J., & Murry, C. E. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology*, 25(9), 1015–1024. <https://doi.org/10.1038/nbt1327>

Laflamme, M. A., & Murry, C. E. (2011). Heart regeneration. In *Nature* (Vol. 473, Issue 7347, pp. 326–335). Nature. <https://doi.org/10.1038/nature10147>

- Lam, C. K., Tian, L., Belbachir, N., Wnorowski, A., Shrestha, R., Ma, N., Kitani, T., Rhee, J. W., & Wu, J. C. (2019). Identifying the transcriptome signatures of calcium channel blockers in human induced pluripotent stem cell-derived cardiomyocytes. *Circulation Research*, 125(2), 212–222. <https://doi.org/10.1161/CIRCRESAHA.118.314202>
- Lan, F., Lee, A. S., Liang, P., Sanchez-Freire, V., Nguyen, P. K., Wang, L., Han, L., Yen, M., Wang, Y., Sun, N., Abilez, O. J., Hu, S., Ebert, A. D., Navarrete, E. G., Simmons, C. S., Wheeler, M., Pruitt, B., Lewis, R., Yamaguchi, Y., ... Wu, J. C. (2013). Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell*, 12(1), 101–113. <https://doi.org/10.1016/j.stem.2012.10.010>
- Lee, Y. K., Ng, K. M., Chan, Y. C., Lai, W. H., Au, K. W., Ho, C. Y. J., Wong, L. Y., Lau, C. P., Tse, H. F., & Siu, C. W. (2010). Triiodothyronine promotes cardiac differentiation and maturation of embryonic stem cells via the classical genomic pathway. *Molecular Endocrinology*, 24(9), 1728–1736. <https://doi.org/10.1210/me.2010-0032>
- LeGrice, I. J., Takayama, Y., & Covell, J. W. (1995). Transverse shear along myocardial cleavage planes provides a mechanism for normal systolic wall thickening. *Circulation Research*, 77(1), 182–193. <https://doi.org/10.1161/01.RES.77.1.182>
- Lehnart, S. E., Wehrens, X. H. T., Reiken, S., Warrier, S., Belevych, A. E., Harvey, R. D., Richter, W., Jin, S. L. C., Conti, M., & Marks, A. R. (2005). Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell*, 123(1), 25–35. <https://doi.org/10.1016/j.cell.2005.07.030>
- Lehnert, D., Wehrle-Haller, B., David, C., Weiland, U., Ballestrem, C., Imhof, B. A., & Bastmeyer, M. (2004). Cell behaviour on micropatterned substrata: Limits of extracellular matrix geometry for spreading and adhesion. *Journal of Cell Science*, 117(1), 41–52. <https://doi.org/10.1242/jcs.00836>
- Lemoine, M. D., Mannhardt, I., Breckwoldt, K., Prondzynski, M., Flenner, F., Ulmer, B., Hirt, M. N., Neuber, C., Horváth, A., Kloth, B., Reichenspurner, H., Willems, S., Hansen, A., Eschenhagen, T., & Christ, T. (2017). Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density. *Scientific Reports*, 7(1), 1–11. <https://doi.org/10.1038/s41598-017-05600-w>
- Leroy, J., Abi-Gerges, A., Nikolaev, V. O., Richter, W., Lechêne, P., Mazet, J.-L., Conti, M., Fischmeister, R., & Vandecasteele, G. (2008). Spatiotemporal dynamics of beta-adrenergic cAMP signals and L-type Ca²⁺ channel regulation in adult rat ventricular myocytes: role of phosphodiesterases. *Circulation Research*, 102(9), 1091–1100. <https://doi.org/10.1161/CIRCRESAHA.107.167817>
- Leroy, J., Richter, W., Mika, D., Castro, L. R. V., Abi-Gerges, A., Xie, M., Scheitrum, C., Lefebvre, F., Schittl, J., Mateo, P., Westenbroek, R., Catterall, W. A., Charpentier, F., Conti, M., Fischmeister, R., & Vandecasteele, G. (2011). Phosphodiesterase 4B in the cardiac L-type Ca²⁺ channel complex regulates Ca²⁺ current and protects against ventricular arrhythmias in mice. *Journal of Clinical Investigation*, 121(7), 2651–2661. <https://doi.org/10.1172/JCI44747>
- Leu, M., Ehler, E., & Perriard, J. C. (2001). Characterisation of postnatal growth of the murine heart. *Anatomy and Embryology*, 204(3), 217–224. <https://doi.org/10.1007/s004290100206>
- Lewandowski, J., Rozwadowska, N., Kolanowski, T. J., Malcher, A., Zimna, A., Rugowska, A., Fiedorowicz, K., Łabędź, W., Kubaszewski, Ł., Chojnacka, K., Bednarek-Rajewska, K., Majewski, P., & Kurpisz, M. (2018). The impact of in vitro cell culture duration on the maturation of human cardiomyocytes

derived from induced pluripotent stem cells of myogenic origin. *Cell Transplantation*, 27(7), 1047–1067. <https://doi.org/10.1177/0963689718779346>

- Li, B., Yang, H., Wang, X., Zhan, Y., Sheng, W., Cai, H., Xin, H., Liang, Q., Zhou, P., Lu, C., Qian, R., Chen, S., Yang, P., Zhang, J., Shou, W., Huang, G., Liang, P., & Sun, N. (2017). Engineering human ventricular heart muscles based on a highly efficient system for purification of human pluripotent stem cell-derived ventricular cardiomyocytes. *Stem Cell Research and Therapy*, 8(1). <https://doi.org/10.1186/s13287-017-0651-x>
- Li, M., Iismaa, S. E., Naqvi, N., Nicks, A., Husain, A., & Graham, R. M. (2014). Thyroid hormone action in postnatal heart development. In *Stem Cell Research* (Vol. 13, Issue 3, pp. 582–591). Elsevier. <https://doi.org/10.1016/j.scr.2014.07.001>
- Lian, X., Zhang, J., Zhu, K., Kamp, T. J., & Palecek, S. P. (2013). Insulin inhibits cardiac mesoderm, not mesendoderm, formation during cardiac differentiation of human pluripotent stem cells and modulation of canonical wnt signaling can rescue this inhibition. *Stem Cells*, 31(3), 447–457. <https://doi.org/10.1002/stem.1289>
- Lieu, A., Lieu, D. K., Liu, J., Siu, C.-W., McNerney, G. P., Tse, H.-F., Abu-Khalil, A., Huser, T., & Li, R. A. (2009). Title Absence of transverse tubules contributes to non-uniform Ca²⁺ wavefronts in mouse and human embryonic stem cell-derived cardiomyocytes Citation Stem Cells Absence of Transverse Tubules Contributes to Non-Uniform Ca²⁺ Wavefronts in Mouse and Human Embryonic Stem Cell-Derived Cardiomyocytes. *And Development*, 10, 1493–1500. <https://doi.org/10.1089/scd.2009.0052>
- Liggett, S. B. (2010). Pharmacogenomics of β 1-Adrenergic Receptor Polymorphisms in Heart Failure. In *Heart Failure Clinics* (Vol. 6, Issue 1, pp. 27–33). NIH Public Access. <https://doi.org/10.1016/j.hfc.2009.08.011>
- Liggett, S. B. (2000). β 2-Adrenergic receptor pharmacogenetics. *American Journal of Respiratory and Critical Care Medicine*, 161(3 II). https://doi.org/10.1164/ajrccm.161.supplement_2.a1q4-10
- Liu, Jianwei, Sun, N., Bruce, M. A., Wu, J. C., & Butte, M. J. (2012). Atomic force mechanobiology of pluripotent stem cell-derived cardiomyocytes. *PLoS ONE*, 7(5). <https://doi.org/10.1371/journal.pone.0037559>
- Liu, Jie, Laksman, Z., & Backx, P. H. (2016). The electrophysiological development of cardiomyocytes. In *Advanced Drug Delivery Reviews* (Vol. 96, pp. 253–273). Elsevier B.V. <https://doi.org/10.1016/j.addr.2015.12.023>
- Liu, Jing, Fu, J. D., Siu, C. W., & Li, R. A. (2007). Functional Sarcoplasmic Reticulum for Calcium Handling of Human Embryonic Stem Cell-Derived Cardiomyocytes: Insights for Driven Maturation. *Stem Cells*, 25(12), 3038–3044. <https://doi.org/10.1634/stemcells.2007-0549>
- Lorent, J. H., Diaz-Rohrer, B., Lin, X., Spring, K., Gorfe, A. A., Levental, K. R., & Levental, I. (2017). Structural determinants and functional consequences of protein affinity for membrane rafts. *Nature Communications*, 8(1), 1–10. <https://doi.org/10.1038/s41467-017-01328-3>
- Lugnier, C, Muller, B., le Bec, A., Beaudry, C., & Rousseau, E. (1993). Characterization of indolidan- and rolipram-sensitive cyclic nucleotide phosphodiesterases in canine and human cardiac microsomal fractions. *Journal of Pharmacology and Experimental Therapeutics*, 265(3).

- Lugnier, Claire. (2006). Cyclic nucleotide phosphodiesterase (PDE) superfamily: A new target for the development of specific therapeutic agents. In *Pharmacology and Therapeutics* (Vol. 109, Issue 3, pp. 366–398). Pergamon. <https://doi.org/10.1016/j.pharmthera.2005.07.003>
- Lundy, S. D., Zhu, W. Z., Regnier, M., & Laflamme, M. A. (2013a). Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells and Development*, 22(14), 1991–2002. <https://doi.org/10.1089/scd.2012.0490>
- Lynch, M. J., Hill, E. v., & Houslay, M. D. (2006). Intracellular Targeting of Phosphodiesterase-4 Underpins Compartmentalized cAMP Signaling. In *Current Topics in Developmental Biology* (Vol. 75, pp. 225–259). Curr Top Dev Biol. [https://doi.org/10.1016/S0070-2153\(06\)75007-4](https://doi.org/10.1016/S0070-2153(06)75007-4)
- Ma, J., Guo, L., Fiene, S. J., Anson, B. D., Thomson, J. A., Kamp, T. J., Kolaja, K. L., Swanson, B. J., & January, C. T. (2011). High purity human-induced pluripotent stem cell-derived cardiomyocytes: Electrophysiological properties of action potentials and ionic currents. *American Journal of Physiology - Heart and Circulatory Physiology*, 301(5). <https://doi.org/10.1152/ajpheart.00694.2011>
- MacDougall, D. A., Agarwal, S. R., Stopford, E. A., Chu, H., Collins, J. A., Longster, A. L., Colyer, J., Harvey, R. D., & Calaghan, S. (2012). Caveolae compartmentalise β 2-adrenoceptor signals by curtailing cAMP production and maintaining phosphatase activity in the sarcoplasmic reticulum of the adult ventricular myocyte. *Journal of Molecular and Cellular Cardiology*, 52(2), 388–400. <https://doi.org/10.1016/j.yjmcc.2011.06.014>
- MacKenzie, S. J., Baillie, G. S., McPhee, I., mac Kenzie, C., Seamons, R., McSorley, T., Millen, J., Beard, M. B., van Heeke, G., & Houslay, M. D. (2002). Long PDE4 cAMP specific phosphodiesterases are activated by protein kinase A-mediated phosphorylation of a single serine residue in Upstream Conserved Region 1 (UCR1). *British Journal of Pharmacology*, 136(3), 421–433. <https://doi.org/10.1038/sj.bjp.0704743>
- Maillet, M., van Berlo, J. H., & Molkentin, J. D. (2013). Molecular basis of physiological heart growth: Fundamental concepts and new players. In *Nature Reviews Molecular Cell Biology* (Vol. 14, Issue 1, pp. 38–48). Nature Publishing Group. <https://doi.org/10.1038/nrm3495>
- Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblyt, N., & Marks, A. R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): Defective regulation in failing hearts. *Cell*, 101(4), 365–376. [https://doi.org/10.1016/S0092-8674\(00\)80847-8](https://doi.org/10.1016/S0092-8674(00)80847-8)
- Maxwell, T. J., Ameyaw, M. M., Pritchard, S., Thornton, N., Folayan, G., Githang'a, J., Indalo, A., Tariq, M., Mobarek, A., Evans, D. A., Ofori-Adjei, D., Templeton, A. R., & McLeod, H. L. (2005). Beta-2 adrenergic receptor genotypes and haplotypes in different ethnic groups. *International Journal of Molecular Medicine*, 16(4), 573–580. <https://doi.org/10.3892/ijmm.16.4.573>
- McBeath, R., Pirone, D. M., Nelson, C. M., Bhadriraju, K., & Chen, C. S. (2004). Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell*, 6(4), 483–495. [https://doi.org/10.1016/S1534-5807\(04\)00075-9](https://doi.org/10.1016/S1534-5807(04)00075-9)
- Mcdevitt, T. C., Angello, J. C., Whitney, M. L., Reinecke, H., Hauschka, S. D., Murry, C. E., & Stayton, P. S. (2002). In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces. *J Biomed Mater Res*, 60, 472–479. <https://doi.org/10.1008/jbm.1292>

- McDevitt, T. C., Laflamme, M. A., & Murry, C. E. (2005). Proliferation of cardiomyocytes derived from human embryonic stem cells is mediated via the IGF/PI 3-kinase/Akt signaling pathway. *Journal of Molecular and Cellular Cardiology*, 39(6), 865–873. <https://doi.org/10.1016/j.yjmcc.2005.09.007>
- Mehel, H., Emons, J., Vettel, C., Wittköpper, K., Seppelt, D., Dewenter, M., Lutz, S., Sossalla, S., Maier, L. S., Lechêne, P., Leroy, J., Lefebvre, F., Varin, A., Eschenhagen, T., Nattel, S., Dobrev, D., Zimmermann, W. H., Nikolaev, V. O., Vandecasteele, G., ... El-Armouche, A. (2013). Phosphodiesterase-2 is up-regulated in human failing hearts and blunts β -adrenergic responses in cardiomyocytes. *Journal of the American College of Cardiology*, 62(17), 1596–1606. <https://doi.org/10.1016/j.jacc.2013.05.057>
- Melax, H., research, T. L.-C., & 1969, undefined. (n.d.). Fine structure of developing and adult intercalated discs in rat heart. *Academic.Oup.Com*. Retrieved April 17, 2020, from <https://academic.oup.com/cardiovasces/article-abstract/3/3/261/373320>
- Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G., & Brown, D. A. (1999). Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *Journal of Biological Chemistry*, 274(6), 3910–3917. <https://doi.org/10.1074/jbc.274.6.3910>
- Merchant, E. E., Johnson, S. W., Nguyen, P., Kang, C., & Mallon, W. K. (2008). Takotsubo cardiomyopathy: a case series and review of the literature. *The Western Journal of Emergency Medicine*, 9(2), 104–111. <http://www.ncbi.nlm.nih.gov/pubmed/19561716>
- Mills, R. J., Titmarsh, D. M., Koenig, X., Parker, B. L., Ryall, J. G., Quaife-Ryan, G. A., Voges, H. K., Hodson, M. P., Ferguson, C., Drowley, L., Plowright, A. T., Needham, E. J., Wang, Q. D., Gregorevic, P., Xin, M., Thomas, W. G., Parton, R. G., Nielsen, L. K., Launikonis, B. S., ... Hudson, J. E. (2017). Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. *Proceedings of the National Academy of Sciences of the United States of America*, 114(40), E8372–E8381. <https://doi.org/10.1073/pnas.1707316114>
- Mirbagheri, M., Adibnia, V., Hughes, B. R., Waldman, S. D., Banquy, X., & Hwang, D. K. (2019). Advanced cell culture platforms: a growing quest for emulating natural tissues. In *Materials Horizons* (Vol. 6, Issue 1, pp. 45–71). Royal Society of Chemistry. <https://doi.org/10.1039/c8mh00803e>
- Mokni, W., Keravis, T., Etienne-Selloum, N., Walter, A., Kane, M. O., Schini-Kerth, V. B., & Lugnier, C. (2010). Concerted regulation of cGMP and cAMP phosphodiesterases in early cardiac hypertrophy induced by angiotensin II. *PLoS ONE*, 5(12). <https://doi.org/10.1371/journal.pone.0014227>
- Molina, C. E., Leroy, J., Richter, W., Xie, M., Scheitrum, C., Lee, I. O., Maack, C., Rucker-Martin, C., Donzeau-Gouge, P., Verde, I., Llach, A., Hove-Madsen, L., Conti, M., Vandecasteele, G., & Fischmeister, R. (2012). Cyclic adenosine monophosphate phosphodiesterase type 4 protects against atrial arrhythmias. *Journal of the American College of Cardiology*, 59(24), 2182–2190. <https://doi.org/10.1016/j.jacc.2012.01.060>
- Mongillo, M., McSorley, T., Evellin, S., Sood, A., Lissandron, V., Terrin, A., Huston, E., Hannawacker, A., Lohse, M. J., Pozzan, T., Houslay, M. D., & Zaccolo, M. (2004). Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. *Circulation Research*, 95(1), 67–75. <https://doi.org/10.1161/01.RES.0000134629.84732.11>
- Mongillo, M., Tocchetti, C. G., Terrin, A., Lissandron, V., Cheung, Y. F., Dostmann, W. R., Pozzan, T., Kass, D. A., Paolocci, N., Houslay, M. D., & Zaccolo, M. (2006). Compartmentalized phosphodiesterase-2

activity blunts β -adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. *Circulation Research*, 98(2), 226–234. <https://doi.org/10.1161/01.RES.0000200178.34179.93>

Moretti, A., Bellin, M., Welling, A., Jung, C. B., Lam, J. T., Bott-Flügel, L., Dorn, T., Goedel, A., Höhnke, C., Hofmann, F., Seyfarth, M., Sinnecker, D., Schömig, A., & Laugwitz, K.-L. (2010). Patient-Specific Induced Pluripotent Stem-Cell Models for Long-QT Syndrome. *New England Journal of Medicine*, 363(15), 1397–1409. <https://doi.org/10.1056/NEJMoa0908679>

Movsesian, M., Ahmad, F., & Hirsch, E. (2018). Functions of PDE3 Isoforms in Cardiac Muscle. *Journal of Cardiovascular Development and Disease*, 5(1), 10. <https://doi.org/10.3390/jcdd5010010>

Mummery, C. L., Zhang, J., Ng, E. S., Elliott, D. A., Elefanty, A. G., & Kamp, T. J. (2012). Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: A methods overview. In *Circulation Research* (Vol. 111, Issue 3, pp. 344–358). <https://doi.org/10.1161/CIRCRESAHA.110.227512>

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., Brutel de la Riviere, A., Passier, R., & Tertoolen, L. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: Role of coculture with visceral endoderm-like cells. *Circulation*, 107(21), 2733–2740. <https://doi.org/10.1161/01.CIR.0000068356.38592.68>

Munakata, M., Harada, Y., Ishida, T., Saito, J., Nagabukuro, A., Matsushita, H., Koga, N., Ohsaki, M., Imagawa, K., & Shiratsuchi, T. (2006). Molecular-based haplotype analysis of the β 2-adrenergic receptor gene (ADRB2) in Japanese asthmatic and non-asthmatic subjects. *Allergology International*, 55(2), 191–198. <https://doi.org/10.2332/allergolint.55.191>

Nakamura, M., & Sadoshima, J. (2018). Mechanisms of physiological and pathological cardiac hypertrophy. In *Nature Reviews Cardiology* (Vol. 15, Issue 7, pp. 387–407). Nature Publishing Group. <https://doi.org/10.1038/s41569-018-0007-y>

Nakano, H., Minami, I., Braas, D., Pappoe, H., Wu, X., Sagadevan, A., Vergnes, L., Fu, K., Morselli, M., Dunham, C., Ding, X., Stieg, A. Z., Gimzewski, J. K., Pellegrini, M., Clark, P. M., Reue, K., Lusic, A. J., Ribalet, B., Kurdistani, S. K., ... Nakano, A. (2017). Glucose inhibits cardiac muscle maturation through nucleotide biosynthesis. *ELife*, 6. <https://doi.org/10.7554/eLife.29330>

Nguyen, P. K., Rhee, J. W., & Wu, J. C. (2016). Adult stem cell therapy and heart failure, 2000 to 2016: A systematic review. In *JAMA Cardiology* (Vol. 1, Issue 7, pp. 831–841). American Medical Association. <https://doi.org/10.1001/jamacardio.2016.2225>

Nichols, C. B., Rossow, C. F., Navedo, M. F., Westenbroek, R. E., Catterall, W. A., Santana, L. F., & McKnight, G. S. (2010). Sympathetic stimulation of adult cardiomyocytes requires association of AKAP5 with a subpopulation of L-Type calcium channels. *Circulation Research*, 107(6), 747–756. <https://doi.org/10.1161/CIRCRESAHA.109.216127>

Niino, Y., Hotta, K., & Oka, K. (2009). Simultaneous live cell imaging using dual FRET sensors with a single excitation light. *PLoS ONE*, 4(6). <https://doi.org/10.1371/journal.pone.0006036>

Nikolaev, V. O., Bünemann, M., Schmitteckert, E., Lohse, M. J., & Engelhardt, S. (2006). Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching β 1-adrenergic but locally confined β 2-adrenergic receptor-mediated signaling. *Circulation Research*, 99(10), 1084–1091. <https://doi.org/10.1161/01.RES.0000250046.69918.d5>

- Nikolaev, V. O., Moshkov, A., Lyon, A. R., Miragoli, M., Novak, P., Paur, H., Lohse, M. J., Korchev, Y. E., Harding, S. E., & Gorelik, J. (2010). β 2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science*. <https://doi.org/10.1126/science.1185988>
- Norman, R., Fuller, W., & Calaghan, S. (2018). Caveolae and the cardiac myocyte. In *Current Opinion in Physiology* (Vol. 1, pp. 59–67). Elsevier Ltd. <https://doi.org/10.1016/j.cophys.2017.08.005>
- Norström, A., Åkesson, K., Hardarson, T., Hamberger, L., Björquist, P., & Sartipy, P. (2006). Molecular and pharmacological properties of human embryonic stem cell-derived cardiomyocytes. *Experimental Biology and Medicine*, 231(11), 1753–1762. <https://doi.org/10.1177/153537020623101113>
- Novak, P., Li, C., Shevchuk, A. I., Stepanyan, R., Caldwell, M., Hughes, S., Smart, T. G., Gorelik, J., Ostanin, V. P., Lab, M. J., Moss, G. W. J., Frolenkov, G. I., Klenerman, D., & Korchev, Y. E. (2009). Nanoscale live-cell imaging using hopping probe ion conductance microscopy. *Nature Methods*, 6(4), 279–281. <https://doi.org/10.1038/nmeth.1306>
- Nussbaum, J., Minami, E., Laflamme, M. A., Virag, J. A. I., Ware, C. B., Masino, A., Muskheli, V., Pabon, L., Reinecke, H., & Murry, C. E. (2007). Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *The FASEB Journal*, 21(7), 1345–1357. <https://doi.org/10.1096/fj.06-6769com>
- Olivetti, G., Cigola, E., Maestri, R., Corradi, D., Lagrasta, C., Gambert, S. R., & Anversa, P. (1996). Aging, cardiac hypertrophy and ischemic cardiomyopathy do not affect the proportion of mononucleated and multinucleated myocytes in the human heart. *Journal of Molecular and Cellular Cardiology*, 28(7), 1463–1477. <https://doi.org/10.1006/jmcc.1996.0137>
- Ostrom, R. S., & Insel, P. A. (2004). The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: Implications for molecular pharmacology. In *British Journal of Pharmacology* (Vol. 143, Issue 2, pp. 235–245). Wiley-Blackwell. <https://doi.org/10.1038/sj.bjp.0705930>
- Ostrom, R. S., Gregorian, C., Drenan, R. M., Xiang, Y., Regan, J. W., & Insel, P. A. (2001). Receptor Number and Caveolar Co-localization Determine Receptor Coupling Efficiency to Adenylyl Cyclase. *Journal of Biological Chemistry*, 276(45), 42063–42069. <https://doi.org/10.1074/jbc.M105348200>
- Ostrom, R. S., Violin, J. D., Coleman, S., & Insel, P. A. (2000). Selective enhancement of β -adrenergic receptor signaling by overexpression of adenylyl cyclase type 6: Colocalization of receptor and adenylyl cyclase in caveolae of cardiac myocytes. *Molecular Pharmacology*, 57(5), 1075–1079.
- Patel, H. H., Murray, F., & Insel, P. A. (2008). Caveolae as Organizers of Pharmacologically Relevant Signal Transduction Molecules. *Annual Review of Pharmacology and Toxicology*, 48(1), 359–391. <https://doi.org/10.1146/annurev.pharmtox.48.121506.124841>
- Paur, H., Wright, P. T., Sikkil, M. B., Tranter, M. H., Mansfield, C., O’Gara, P., Stuckey, D. J., Nikolaev, V. O., Diakonov, I., Pannell, L., Gong, H., Sun, H., Peters, N. S., Petrou, M., Zheng, Z., Gorelik, J., Lyon, A. R., & Harding, S. E. (2012). High levels of circulating epinephrine trigger apical cardiodepression in a β 2-adrenergic receptor/Gi-dependent manner: A new model of takotsubo cardiomyopathy. *Circulation*, 126(6), 697–706. <https://doi.org/10.1161/CIRCULATIONAHA.112.111591>
- Pavlaki, N., & Nikolaev, V. (2018). Imaging of PDE2- and PDE3-Mediated cGMP-to-cAMP Cross-Talk in Cardiomyocytes. *Journal of Cardiovascular Development and Disease*. <https://doi.org/10.3390/jcdd5010004>

- Perera, R. K., Sprenger, J. U., Steinbrecher, J. H., Hübscher, D., Lehnart, S. E., Abesser, M., Schuh, K., El-Armouche, A., & Nikolaev, V. O. (2015). Microdomain switch of cGMP-regulated phosphodiesterases leads to ANP-induced augmentation of β -adrenoceptor-stimulated contractility in early cardiac hypertrophy. *Circulation Research*, 116(8), 1304–1311. <https://doi.org/10.1161/CIRCRESAHA.116.306082>
- Pesl, M., Pribyl, J., Caluori, G., Cmiel, V., Acimovic, I., Jelinkova, S., Dvorak, P., Starek, Z., Skladal, P., & Rotrekl, V. (2017). Phenotypic assays for analyses of pluripotent stem cell-derived cardiomyocytes. *Journal of Molecular Recognition*, 30(6), e2602. <https://doi.org/10.1002/jmr.2602>
- Peters, N S, Green, C. R., Poole-Wilson, P. A., & Severs, N. J. (1993). Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. *Circulation*, 88(3), 864–875. <https://doi.org/10.1161/01.CIR.88.3.864>
- Peters, Nicholas S., Severs, N. J., Rothery, S. M., Lincoln, C., Yacoub, M. H., & Green, C. R. (1994). Spatiotemporal relation between gap junctions and fascia adherens junctions during postnatal development of human ventricular myocardium. *Circulation*, 90(2), 713–725. <https://doi.org/10.1161/01.CIR.90.2.713>
- Pierce, K. L., Premont, R. T., & Lefkowitz, R. J. (2002). Seven-transmembrane receptors. In *Nature Reviews Molecular Cell Biology* (Vol. 3, Issue 9, pp. 639–650). Nature Publishing Group. <https://doi.org/10.1038/nrm908>
- Pillekamp, F., Hausteil, M., Khalil, M., Emmelheinz, M., Nazzal, R., Adelmann, R., Nguemo, F., Rubenchyk, O., Pfannkuche, K., Matzkies, M., Reppel, M., Bloch, W., Brockmeier, K., & Hescheler, J. (2012). Contractile properties of early human embryonic stem cell-derived cardiomyocytes: Beta-adrenergic stimulation induces positive chronotropy and lusitropy but not inotropy. *Stem Cells and Development*, 21(12), 2111–2121. <https://doi.org/10.1089/scd.2011.0312>
- Porrello, E. R., Mahmoud, A. I., Simpson, E., Hill, J. A., Richardson, J. A., Olson, E. N., & Sadek, H. A. (2011). Transient regenerative potential of the neonatal mouse heart. *Science*, 331(6020), 1078–1080. <https://doi.org/10.1126/science.1200708>
- Prajapati, C., Ojala, M., & Aalto-Setälä, K. (2018). Divergent effects of adrenaline in human induced pluripotent stem cell-derived cardiomyocytes obtained from hypertrophic cardiomyopathy. *DMM Disease Models and Mechanisms*, 11(2). <https://doi.org/10.1242/DMM.032896>
- Qu, Y., & Boutjdir, M. (2001). Gene expression of SERCA2a and L- and T-type Ca channels during human heart development. *Pediatric Research*, 50(5), 569–574. <https://doi.org/10.1203/00006450-200111000-00006>
- Radisic, M., Euloth, M., Yang, L., Langer, R., Freed, L. E., & Vunjak-Novakovic, G. (2003). High-density seeding of myocyte cells for cardiac tissue engineering. *Biotechnology and Bioengineering*, 82(4), 403–414. <https://doi.org/10.1002/bit.10594>
- Rajagopal, S., & Shenoy, S. K. (2018). GPCR desensitization: Acute and prolonged phases. In *Cellular Signalling* (Vol. 41, pp. 9–16). Elsevier Inc. <https://doi.org/10.1016/j.cellsig.2017.01.024>
- Ramuz, M., Hasan, A., Gruscheski, L., Diakonov, I., Pavlaki, N., Nikolaev, V. O., Harding, S., Dunsby, C., & Gorelik, J. (2019). A Software Tool for High-Throughput Real-Time Measurement of Intensity-Based Ratio-Metric FRET. *Cells*, 8(12), 1541. <https://doi.org/10.3390/cells8121541>

- Rao, C., Prodromakis, T., Kolker, L., Chaudhry, U. A. R., Trantidou, T., Sridhar, A., Weekes, C., Camelliti, P., Harding, S. E., Darzi, A., Yacoub, M. H., Athanasiou, T., & Terracciano, C. M. (2013). The effect of microgrooved culture substrates on calcium cycling of cardiac myocytes derived from human induced pluripotent stem cells. *Biomaterials*, 34(10), 2399–2411. <https://doi.org/10.1016/j.biomaterials.2012.11.055>
- Reinecke, H., & Murry, C. E. (2000). Transmural replacement of myocardium after skeletal myoblast grafting into the heart: Too much of a good thing? *Cardiovascular Pathology*, 9(6), 337–344. [https://doi.org/10.1016/S1054-8807\(00\)00055-7](https://doi.org/10.1016/S1054-8807(00)00055-7)
- Reinecke, H., Poppa, V., & Murry, C. E. (2002). Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. *Journal of Molecular and Cellular Cardiology*, 34(2), 241–249. <https://doi.org/10.1006/jmcc.2001.1507>
- Reppel, M., Boettinger, C., & Hescheler, J. (2004). Beta-adrenergic and muscarinic modulation of human embryonic stem cell-derived cardiomyocytes. *Cellular Physiology and Biochemistry*, 14(4–6), 187–196. <https://doi.org/10.1159/000080326>
- Ribeiro, A. J. S., Ang, Y.-S., Fu, J.-D., Rivas, R. N., Mohamed, T. M. A., Higgs, G. C., Srivastava, D., & Pruitt, B. L. (2015). Contractility of single cardiomyocytes differentiated from pluripotent stem cells depends on physiological shape and substrate stiffness. *Proceedings of the National Academy of Sciences*. <https://doi.org/10.1073/pnas.1508073112>
- Richards, D. J., Tan, Y., Coyle, R., Li, Y., Xu, R., Yeung, N., Parker, A., Menick, D. R., Tian, B., & Mei, Y. (2016). Nanowires and electrical stimulation synergistically improve functions of hiPSC cardiac spheroids. *Nano Letters*, 16(7), 4670–4678. <https://doi.org/10.1021/acs.nanolett.6b02093>
- Richter, W., Day, P., Agrawal, R., Bruss, M. D., Granier, S., Wang, Y. L., Rasmussen, S. G. F., Horner, K., Wang, P., Lei, T., Patterson, A. J., Kobilka, B., & Conti, M. (2008a). Signaling from β 1- and β 2-adrenergic receptors is defined by differential interactions with PDE4. *EMBO Journal*. <https://doi.org/10.1038/sj.emboj.7601968>
- Richter, W., Day, P., Agrawal, R., Bruss, M. D., Granier, S., Wang, Y. L., Rasmussen, S. G. F., Horner, K., Wang, P., Lei, T., Patterson, A. J., Kobilka, B., & Conti, M. (2008b). Signaling from β 1- and β 2-adrenergic receptors is defined by differential interactions with PDE4. *EMBO Journal*, 27(2), 384–393. <https://doi.org/10.1038/sj.emboj.7601968>
- Richter, W., Mika, D., Blanchard, E., Day, P., & Conti, M. (2013). β 1-adrenergic receptor antagonists signal via PDE4 translocation. *EMBO Reports*, 14(3), 276–283. <https://doi.org/10.1038/embor.2013.4>
- Richter, W., Xie, M., Scheitrum, C., Krall, J., Movsesian, M. A., & Conti, M. (2011). Conserved expression and functions of PDE4 in rodent and human heart. *Basic Research in Cardiology*. <https://doi.org/10.1007/s00395-010-0138-8>
- Rochais, F., Vandecasteele, G., Lefebvre, F., Lugnier, C., Lum, H., Mazet, J. L., Cooper, D. M. F., & Fischmeister, R. (2004). Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact cardiac myocytes: An in vivo study using adenovirus-mediated expression of CNG channels. *Journal of Biological Chemistry*, 279(50), 52095–52105. <https://doi.org/10.1074/jbc.M405697200>
- Rockman, H. A., Koch, W. J., & Lefkowitz, R. J. (2002). Seven-transmembrane-spanning receptors and heart function. In *Nature* (Vol. 415, Issue 6868, pp. 206–212). Nature Publishing Group. <https://doi.org/10.1038/415206a>

- Ronaldson-Bouchard, K., Ma, S. P., Yeager, K., Chen, T., Song, L. J., Sirabella, D., Morikawa, K., Teles, D., Yazawa, M., & Vunjak-Novakovic, G. (2018a). Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature*, 556(7700), 239–243. <https://doi.org/10.1038/s41586-018-0016-3>
- Ronaldson-Bouchard, K., Ma, S. P., Yeager, K., Chen, T., Song, L. J., Sirabella, D., Morikawa, K., Teles, D., Yazawa, M., & Vunjak-Novakovic, G. (2018b). Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature*, 556(7700), 239–243. <https://doi.org/10.1038/s41586-018-0016-3>
- Rosenbaum, D. M., Rasmussen, S. G. F., & Kobilka, B. K. (2009). The structure and function of G-protein-coupled receptors. In *Nature* (Vol. 459, Issue 7245, pp. 356–363). NIH Public Access. <https://doi.org/10.1038/nature08144>
- Roshanbinfar, K., Mohammadi, Z., Sheikh-Mahdi Mesgar, A., Dehghan, M. M., Oommen, O. P., Hilborn, J., & Engel, F. B. (2019). Carbon nanotube doped pericardial matrix derived electroconductive biohybrid hydrogel for cardiac tissue engineering. *Biomaterials Science*, 7(9), 3906–3917. <https://doi.org/10.1039/c9bm00434c>
- Rupert, C. E., & Coulombe, K. L. K. (2017). IGF1 and NRG1 Enhance Proliferation, Metabolic Maturity, and the Force-Frequency Response in hESC-Derived Engineered Cardiac Tissues. *Hindawi.Com*. <https://doi.org/10.1155/2017/7648409>
- Rusconi, F., Ceriotti, P., Miragoli, M., Carullo, P., Salvarani, N., Rocchetti, M., di Pasquale, E., Rossi, S., Tessari, M., Caprari, S., Cazade, M., Kunderfranco, P., Chemin, J., Bang, M. L., Polticelli, F., Zaza, A., Faggian, G., Condorelli, G., & Catalucci, D. (2016). Peptidomimetic Targeting of Cav β 2 Overcomes Dysregulation of the L-Type Calcium Channel Density and Recovers Cardiac Function. *Circulation*, 134(7), 534–546. <https://doi.org/10.1161/CIRCULATIONAHA.116.021347>
- Rybin, V. O., Xu, X., Lisanti, M. P., & Steinberg, S. F. (2000). Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *The Journal of Biological Chemistry*, 275(52), 41447–41457. <https://doi.org/10.1074/jbc.M006951200>
- Salick, M. R., Napiwocki, B. N., Sha, J., Knight, G. T., Chindhy, S. A., Kamp, T. J., Ashton, R. S., & Crone, W. C. (2014). Micropattern width dependent sarcomere development in human ESC-derived cardiomyocytes. *Biomaterials*, 35(15), 4454–4464. <https://doi.org/10.1016/j.biomaterials.2014.02.001>
- Samuels, M. A. (2007). The brain-heart connection. *Circulation*, 116(1), 77–84. <https://doi.org/10.1161/CIRCULATIONAHA.106.678995>
- Sanger, J. W., Ayoob, J. C., Chowrashi, P., Zurawski, D., Sanger, J. M., Baatsen, Keller, Pollack, & Greaser. (2000). Assembly of myofibrils in cardiac muscle cells. *Advances in Experimental Medicine and Biology*, 481, 89–105. https://doi.org/10.1007/978-1-4615-4267-4_6
- Sattler, S., Couch, L. S., & Harding, S. E. (2018). Takotsubo Syndrome: Latest Addition to the Expanding Family of Immune-Mediated Diseases? In *JACC: Basic to Translational Science* (Vol. 3, Issue 6, pp. 779–781). Elsevier Inc. <https://doi.org/10.1016/j.jacbts.2018.11.003>
- Sato H, Tateishi H, Uchida T, Ai E. (1990). Takotsubo-type cardiomyopathy due to multivessel spasm. *Clinical Aspects of Myocardial Injury: From Ischemia to Heart Failure*. Tokyo: Japan

Kagakuhyouronsha, 1990:56–64
<https://ci.nii.ac.jp/naid/10030035349/>

- Schobesberger, S., Wright, P., Tokar, S., Bhargava, A., Mansfield, C., Glukhov, A. v., Poulet, C., Buzuk, A., Monzpart, A., Sikkell, M., Harding, S. E., Nikolaev, V. O., Lyon, A. R., & Gorelik, J. (2017). T-tubule remodelling disturbs localized β 2-adrenergic signalling in rat ventricular myocytes during the progression of heart failure. *Cardiovascular Research*, 113(7), 770–782. <https://doi.org/10.1093/cvr/cvx074>
- Schwenzer, N., Bussmann, H., Franken, S., & Haeberlein, H. (2018). Live-cell single-molecule analysis of β 2-adrenergic receptor diffusion dynamics and confinement. *BioRxiv*, 3, 406488. <https://doi.org/10.1101/406488>
- Scriven, D. R. L., Dan, P., & Moore, E. D. W. (2000). Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. *Biophysical Journal*, 79(5), 2682–2691. [https://doi.org/10.1016/S0006-3495\(00\)76506-4](https://doi.org/10.1016/S0006-3495(00)76506-4)
- Scuderi, G. J., & Butcher, J. (2017a). Naturally engineered maturation of cardiomyocytes. In *Frontiers in Cell and Developmental Biology* (Vol. 5, Issue MAY). Frontiers Media S.A. <https://doi.org/10.3389/fcell.2017.00050>
- Sette, C., & Conti, M. (1996). Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. *Journal of Biological Chemistry*, 271(28), 16526–16534. <https://doi.org/10.1074/jbc.271.28.16526>
- Severs, N. J., Bruce, A. F., Dupont, E., & Rothery, S. (n.d.). *Remodelling of gap junctions and connexin expression in diseased myocardium*. <https://doi.org/10.1093/cvr/cvn133>
- Shakur, Y., Holst, L. S., Landstrom, T. R., Movsesian, M., Degerman, E., & Manganiello, V. (2000). Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family. In *Progress in Nucleic Acid Research and Molecular Biology* (Vol. 66, pp. 241–277). Prog Nucleic Acid Res Mol Biol. [https://doi.org/10.1016/s0079-6603\(00\)66031-2](https://doi.org/10.1016/s0079-6603(00)66031-2)
- Shaw, R. M., & Colecraft, H. M. (n.d.). *L-type calcium channel targeting and local signalling in cardiac myocytes*. <https://doi.org/10.1093/cvr/cvt021>
- Sheikh, F., Ross, R. S., & Chen, J. (2009). Cell-Cell Connection to Cardiac Disease. In *Trends in Cardiovascular Medicine* (Vol. 19, Issue 6, pp. 182–190). Trends Cardiovasc Med. <https://doi.org/10.1016/j.tcm.2009.12.001>
- Shenoy, S. K., & Lefkowitz, R. J. (2011). β -arrestin-mediated receptor trafficking and signal transduction. In *Trends in Pharmacological Sciences* (Vol. 32, Issue 9, pp. 521–533). <https://doi.org/10.1016/j.tips.2011.05.002>
- Shiba, Y., Gomibuchi, T., Seto, T., Wada, Y., Ichimura, H., Tanaka, Y., Ogasawara, T., Okada, K., Shiba, N., Sakamoto, K., Ido, D., Shiina, T., Ohkura, M., Nakai, J., Uno, N., Kazuki, Y., Oshimura, M., Minami, I., & Ikeda, U. (2016). Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature*, 538(7625), 388–391. <https://doi.org/10.1038/nature19815>
- Shintani, Y., Fukushima, S., Varela-Carver, A., Lee, J., Coppen, S. R., Takahashi, K., Brouillette, S. W., Yashiro, K., Terracciano, C. M. N., Yacoub, M. H., & Suzuki, K. (2009). Donor cell-type specific paracrine effects of cell transplantation for post-infarction heart failure. *Journal of Molecular and Cellular Cardiology*, 47(2), 288–295. <https://doi.org/10.1016/j.yjmcc.2009.05.009>

- Silbernagel, N., Körner, A., Balitzki, J., Jaggy, M., Bertels, S., Richter, B., Hippler, M., Hellwig, A., Hecker, M., Bastmeyer, M., & Ullrich, N. D. (2020). Shaping the heart: Structural and functional maturation of iPSC-cardiomyocytes in 3D-micro-scaffolds. *Biomaterials*, 227, 119551. <https://doi.org/10.1016/j.biomaterials.2019.119551>
- Singer, S. J., & Nicolson, G. L. (1972). The fluid mosaic model of the structure of cell membranes. *Science*, 175(4023), 720–731. <https://doi.org/10.1126/science.175.4023.720>
- Sinnecker, D., Dirschinger, R. J., Goedel, A., Moretti, A., Lipp, P., & Laugwitz, K.-L. (2012). Induced Pluripotent Stem Cells in Cardiovascular Research. In *Reviews of Physiology, Biochemistry and Pharmacology*, Vol. 163 (pp. 1–26). Springer Berlin Heidelberg. https://doi.org/10.1007/112_2012_6
- Slotkin, T. A., Lau, C., & Seidler, F. J. (1994). β -adrenergic receptor overexpression in the fetal rat: Distribution, receptor subtypes, and coupling to adenylate cyclase activity via G-proteins. *Toxicology and Applied Pharmacology*, 129(2), 223–234. <https://doi.org/10.1006/taap.1994.1247>
- Smolich, J. J. (1995). Ultrastructural and functional features of the developing mammalian heart: A brief overview. *Reproduction, Fertility and Development*, 7(3), 451–461. <https://doi.org/10.1071/RD9950451>
- Snir, M., Kehat, I., Gepstein, A., Coleman, R., Itskovitz-Eldor, J., Livne, E., & Gepstein, L. (2003a). Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. *American Journal of Physiology - Heart and Circulatory Physiology*, 285(6 54-6). <https://doi.org/10.1152/ajpheart.00020.2003>
- Stangherlin, A., Gesellchen, F., Zoccarato, A., Terrin, A., Fields, L. A., Berrera, M., Surdo, N. C., Craig, M. A., Smith, G., Hamilton, G., & Zaccolo, M. (2011). CGMP signals modulate camp levels in a compartment-specific manner to regulate catecholamine-dependent signaling in cardiac myocytes. *Circulation Research*, 108(8), 929–939. <https://doi.org/10.1161/CIRCRESAHA.110.230698>
- Stangherlin, A., & Zaccolo, M. (2011). Local Termination of 3'-5'-Cyclic Adenosine Monophosphate Signals: The Role of A Kinase Anchoring Protein–Tethered Phosphodiesterases. *Journal of Cardiovascular Pharmacology*, 58(4), 345–353. <https://doi.org/10.1097/FJC.0b013e3182214f2b>
- Steinberg, S. F. (1999). The Molecular Basis for Distinct β -Adrenergic Receptor Subtype Actions in Cardiomyocytes. *Circulation Research*, 85(11), 1101–1111. <https://doi.org/10.1161/01.RES.85.11.1101>
- Steinberg, S. F., & Brunton, L. L. (2001). COMPARTMENTATION OF G PROTEIN -COUPLED SIGNALING PATHWAYS IN CARDIAC MYOCYTES. *Annual Review of Pharmacology and Toxicology*, 41(1), 751–773. <https://doi.org/10.1146/annurev.pharmtox.41.1.751>
- Steinhoff, G., Nesteruk, J., Wolfien, M., Große, J., Ruch, U., Vasudevan, P., & Müller, P. (2017). Stem cells and heart disease - Brake or accelerator? In *Advanced Drug Delivery Reviews* (Vol. 120, pp. 2–24). Elsevier B.V. <https://doi.org/10.1016/j.addr.2017.10.007>
- Sun, B., Li, H., Shakur, Y., Hensley, J., Hockman, S., Kambayashi, J., Manganiello, V. C., & Liu, Y. (2007). Role of phosphodiesterase type 3A and 3B in regulating platelet and cardiac function using subtype-selective knockout mice. *Cellular Signalling*, 19(8), 1765–1771. <https://doi.org/10.1016/j.cellsig.2007.03.012>
- Sun, N., Yazawa, M., Liu, J., Han, L., Sanchez-Freire, V., Abilez, O. J., Navarrete, E. G., Hu, S., Wang, L., Lee, A., Pavlovic, A., Lin, S., Chen, R., Hajjar, R. J., Snyder, M. P., Dolmetsch, R. E., Butte, M. J., Ashley, E.

- A., Longaker, M. T., ... Wu, J. C. (2012). Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Science Translational Medicine*, 4(130), 130ra47-130ra47. <https://doi.org/10.1126/scitranslmed.3003552>
- Sunyer, T., Monastirsky, B., Codina, J., & Birnbaumer, L. (1989). Studies on Nucleotide and Receptor Regulation of G_i Proteins: Effects of Pertussis Toxin. *Molecular Endocrinology*, 3(7), 1115–1124. <https://doi.org/10.1210/mend-3-7-1115>
- Surdo, N. C., Berrera, M., Koschinski, A., Brescia, M., Machado, M. R., Carr, C., Wright, P., Gorelik, J., Morotti, S., Grandi, E., Bers, D. M., Pantano, S., & Zaccolo, M. (2017). FRET biosensor uncovers cAMP nano-domains at β -adrenergic targets that dictate precise tuning of cardiac contractility. *Nature Communications*, 8, 15031. <https://doi.org/10.1038/ncomms15031>
- Synnergren, J., Améen, C., Jansson, A., & Sartipy, P. (2012). Global transcriptional profiling reveals similarities and differences between human stem cell-derived cardiomyocyte clusters and heart tissue. *Physiological Genomics*, 44(4), 245–258. <https://doi.org/10.1152/physiolgenomics.00118.2011>
- Taggart, P., Critchley, H., van Duijvendoden, S., & Lambiase, P. D. (2016). Significance of neuro-cardiac control mechanisms governed by higher regions of the brain. In *Autonomic Neuroscience: Basic and Clinical* (Vol. 199, pp. 54–65). Elsevier B.V. <https://doi.org/10.1016/j.autneu.2016.08.013>
- Tan, Y., Richards, D., Xu, R., Stewart-Clark, S., Mani, S. K., Borg, T. K., Menick, D. R., Tian, B., & Mei, Y. (2015). Silicon nanowire-induced maturation of cardiomyocytes derived from human induced pluripotent stem cells. *Nano Letters*, 15(5), 2765–2772. <https://doi.org/10.1021/nl502227a>
- Taskén, K. A., Collas, P., Kemmner, W. A., Witczak, O., Conti, M., & Taskén, K. (2001). Phosphodiesterase 4D and Protein Kinase A Type II Constitute a Signaling Unit in the Centrosomal Area. *Journal of Biological Chemistry*, 276(25), 21999–22002. <https://doi.org/10.1074/jbc.C000911200>
- Terrin, A., di Benedetto, G., Pertegato, V., Cheung, Y. F., Baillie, G., Lynch, M. J., Elvassore, N., Prinz, A., Herberg, F. W., Houslay, M. D., & Zaccolo, M. (2006). PGE1 stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: Role of compartmentalized phosphodiesterases. *Journal of Cell Biology*, 175(3), 441–451. <https://doi.org/10.1083/jcb.200605050>
- Thomas, C. H., Collier, J. H., Sfeir, C. S., & Healy, K. E. (2002). Engineering gene expression and protein synthesis by modulation of nuclear shape. *Proceedings of the National Academy of Sciences of the United States of America*, 99(4), 1972–1977. <https://doi.org/10.1073/pnas.032668799>
- Thomson, J. A. (1998). Embryonic stem cell lines derived from human blastocysts. *Science*, 282(5391), 1145–1147. <https://doi.org/10.1126/science.282.5391.1145>
- Tolkovsky, A. M., & Levitzki, A. (1978). Mode of Coupling between the β - Adrenergic Receptor and Adenylate Cyclase in Turkey Erythrocytes. *Biochemistry*, 17(18), 3795–3810. <https://doi.org/10.1021/bi00611a020>
- Toya, Y., Schwencke, C., Couet, J., Lisanti, M. P., & Ishikawa, Y. (1998). Inhibition of Adenylyl Cyclase by Caveolin Peptides¹. *Endocrinology*, 139(4), 2025–2031. <https://doi.org/10.1210/endo.139.4.5957>
- Troncoso, R., Ibarra, C., Vicencio, J. M., Jaimovich, E., & Lavandero, S. (2014). New insights into IGF-1 signaling in the heart. In *Trends in Endocrinology and Metabolism* (Vol. 25, Issue 3, pp. 128–137). Elsevier Current Trends. <https://doi.org/10.1016/j.tem.2013.12.002>

- Trulock, E. P., Christie, J. D., Edwards, L. B., Boucek, M. M., Aurora, P., Taylor, D. O., Dobbels, F., Rahmel, A. O., Keck, B. M., & Hertz, M. I. (2007). Registry of the International Society for Heart and Lung Transplantation: Twenty-fourth Official Adult Lung and Heart-Lung Transplantation Report-2007. *Journal of Heart and Lung Transplantation*, 26(8), 782–795.
<https://doi.org/10.1016/j.healun.2007.06.003>
- Tulloch, N. L., Muskheli, V., Razumova, M. v., Korte, F. S., Regnier, M., Hauch, K. D., Pabon, L., Reinecke, H., & Murry, C. E. (2011). Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circulation Research*, 109(1), 47–59.
<https://doi.org/10.1161/CIRCRESAHA.110.237206>
- Ulmer, B. M., Stoehr, A., Schulze, M. L., Patel, S., Gucek, M., Mannhardt, I., Funcke, S., Murphy, E., Eschenhagen, T., & Hansen, A. (2018). Contractile Work Contributes to Maturation of Energy Metabolism in hiPSC-Derived Cardiomyocytes. *Stem Cell Reports*, 10(3), 834–847.
<https://doi.org/10.1016/j.stemcr.2018.01.039>
- Uzun, A. U., Mannhardt, I., Breckwoldt, K., Horváth, A., Johannsen, S. S., Hansen, A., Eschenhagen, T., & Christ, T. (2016). Ca²⁺-Currents in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Effects of Two Different Culture Conditions. *Frontiers in Pharmacology*, 7(SEP), 300.
<https://doi.org/10.3389/fphar.2016.00300>
- van Gestel, R. A., Brewis, I. A., Ashton, P. R., Helms, J. B., Brouwers, J. F., & Gadella, B. M. (2005). Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. *Molecular Human Reproduction*, 11(8), 583–590. <https://doi.org/10.1093/molehr/gah200>
- Vandeput, F., Wolda, S. L., Krall, J., Hambleton, R., Uher, L., McCaw, K. N., Radwanski, P. B., Florio, V., & Movsesian, M. A. (2007). Cyclic nucleotide phosphodiesterase PDE1C1 in human cardiac myocytes. *Journal of Biological Chemistry*, 282(45), 32749–32757. <https://doi.org/10.1074/jbc.M703173200>
- Veerman, C. C., Kosmidis, G., Mummery, C. L., Casini, S., Verkerk, A. O., & Bellin, M. (2015). Immaturity of Human Stem-Cell-Derived Cardiomyocytes in Culture: Fatal Flaw or Soluble Problem? In *Stem Cells and Development* (Vol. 24, Issue 9, pp. 1035–1052). Mary Ann Liebert Inc.
<https://doi.org/10.1089/scd.2014.0533>
- Veerman, C. C., Mengarelli, I., Lodder, E. M., Kosmidis, G., Bellin, M., Zhang, M., Dittmann, S., Guan, K., Wilde, A. A. M., Schulze-Bahr, E., Greber, B., Bezzina, C. R., & Verkerk, A. O. (2017). Switch From Fetal to Adult SCN5A Isoform in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Unmasks the Cellular Phenotype of a Conduction Disease-Causing Mutation. *Journal of the American Heart Association*, 6(7). <https://doi.org/10.1161/JAHA.116.005135>
- Vidarsson, H., Hyllner, J., & Sartipy, P. (2010). Differentiation of human embryonic stem cells to cardiomyocytes for in vitro and in vivo applications. In *Stem Cell Reviews and Reports* (Vol. 6, Issue 1, pp. 108–120). Springer. <https://doi.org/10.1007/s12015-010-9113-x>
- Vistein, R., & Puthenveedu, M. A. (2013). Reprogramming of G protein-coupled receptor recycling and signaling by a kinase switch. *Proceedings of the National Academy of Sciences of the United States of America*, 110(38), 15289–15294. <https://doi.org/10.1073/pnas.1306340110>
- Wang, J., Gareri, C., & Rockman, H. A. (2018). G-protein-coupled receptors in heart disease. In *Circulation Research* (Vol. 123, Issue 6, pp. 716–735). Lippincott Williams and Wilkins.
<https://doi.org/10.1161/CIRCRESAHA.118.311403>

- Wang, P. Y., Yu, J., Lin, J. H., & Tsai, W. B. (2011). Modulation of alignment, elongation and contraction of cardiomyocytes through a combination of nanotopography and rigidity of substrates. *Acta Biomaterialia*, 7(9), 3285–3293. <https://doi.org/10.1016/j.actbio.2011.05.021>
- Way, M., & Parton, R. G. (1995). M-caveolin, a muscle-specific caveolin-related protein. *FEBS Letters*, 376(1–2), 108–112. [https://doi.org/10.1016/0014-5793\(95\)01256-7](https://doi.org/10.1016/0014-5793(95)01256-7)
- Wechsler, J., Choi, Y. H., Krall, J., Ahmad, F., Manganiello, V. C., & Movsesian, M. A. (2002a). Isoforms of cyclic nucleotide phosphodiesterase PDE3A in cardiac myocytes. *Journal of Biological Chemistry*, 277(41), 38072–38078. <https://doi.org/10.1074/jbc.M203647200>
- Wehrens, X. H. T., Lehnart, S. E., Huang, F., Vest, J. A., Reiken, S. R., Mohler, P. J., Sun, J., Guatimosim, S., Song, L. S., Rosemblyt, N., D’Armiento, J. M., Napolitano, C., Memmi, M., Priori, S. G., Lederer, W. J., & Marks, A. R. (2003). FKBP12.6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death. *Cell*, 113(7), 829–840. [https://doi.org/10.1016/S0092-8674\(03\)00434-3](https://doi.org/10.1016/S0092-8674(03)00434-3)
- Wei, B., & Jin, J. P. (2016). TNNT1, TNNT2, and TNNT3: Isoform genes, regulation, and structure-function relationships. In *Gene* (Vol. 582, Issue 1, pp. 1–13). Elsevier B.V. <https://doi.org/10.1016/j.gene.2016.01.006>
- Williams, T. M., & Lisanti, M. P. (2004). The caveolin proteins. In *Genome Biology* (Vol. 5, Issue 3, p. 214). BioMed Central. <https://doi.org/10.1186/gb-2004-5-3-214>
- Wittstein, I. S., Thiemann, D. R., Lima, J. A. C., Baughman, K. L., Schulman, S. P., Gerstenblith, G., Wu, K. C., Rade, J. J., Bivalacqua, T. J., & Champion, H. C. (2005). Neurohumoral features of myocardial stunning due to sudden emotional stress. *New England Journal of Medicine*, 352(6), 539–548. <https://doi.org/10.1056/NEJMoa043046>
- Wright, P. T., Nikolaev, V. O., O’Hara, T., Diakonov, I., Bhargava, A., Tokar, S., Schobesberger, S., Shevchuk, A. I., Sikkil, M. B., Wilkinson, R., Trayanova, N. A., Lyon, A. R., Harding, S. E., & Gorelik, J. (2014). Caveolin-3 regulates compartmentation of cardiomyocyte beta2-adrenergic receptor-mediated cAMP signaling. *Journal of Molecular and Cellular Cardiology*, 67, 38–48. <https://doi.org/10.1016/j.yjmcc.2013.12.003>
- Wu, H., Lee, J., Gu, M., Lan, F., Churko, J., Sanchez-Freire, V., Matsa, E., Ebert, A., Sallam, K., Gold, J., & Wu, J. (2014). Epigenetic activation of phosphodiesterase subtypes lead to compromised beta-adrenergic signaling in induced pluripotent stem cell-derived cardiomyocytes from dilated cardiomyopathy patients. *Circulation*.
- Wu, Haodi, Lee, J., Vincent, L. G., Wang, Q., Gu, M., Lan, F., Churko, J. M., Sallam, K. I., Matsa, E., Sharma, A., Gold, J. D., Engler, A. J., Xiang, Y. K., Bers, D. M., & Wu, J. C. (2015). Epigenetic Regulation of Phosphodiesterases 2A and 3A Underlies Compromised β -Adrenergic Signaling in an iPSC Model of Dilated Cardiomyopathy. *Cell Stem Cell*. <https://doi.org/10.1016/j.stem.2015.04.020>
- Wypijewski, K. J., Tinti, M., Chen, W., Lamont, D., Ashford, M. L. J., Calaghan, S. C., & Fuller, W. (2015). Identification of caveolar resident proteins in ventricular myocytes using a quantitative proteomic approach: Dynamic changes in caveolar composition following adrenoceptor activation. *Molecular and Cellular Proteomics*, 14(3), 596–608. <https://doi.org/10.1074/mcp.M114.038570>
- Xiang, Y., & Kobilka, B. (2003a). The PDZ-binding motif of the β 2-adrenoceptor is essential for physiologic signaling and trafficking in cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 100(19), 10776–10781. <https://doi.org/10.1073/pnas.1831718100>

- Xiang, Y., & Kobilka, B. K. (2003b). Myocyte adrenoceptor signaling pathways. In *Science* (Vol. 300, Issue 5625, pp. 1530–1532). American Association for the Advancement of Science. <https://doi.org/10.1126/science.1079206>
- Xiang, Y., Naro, F., Zoudilova, M., Jin, S. L. C., Conti, M., & Kobilka, B. (2005). Phosphodiesterase 4D is required for β_2 adrenoceptor subtype-specific signaling in cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 102(3), 909–914. <https://doi.org/10.1073/pnas.0405263102>
- Xiang, Y., Rybin, V. O., Steinberg, S. F., & Kobilka, B. (2002). Caveolar localization dictates physiologic signaling of β_2 -adrenoceptors in neonatal cardiac myocytes. *Journal of Biological Chemistry*, 277(37), 34280–34286. <https://doi.org/10.1074/jbc.M201644200>
- Xiao, R. P., Hohl, C., Altschuld, R., Jones, L., Livingston, B., Ziman, B., Tantini, B., & Lakatta, E. G. (1994). Beta 2-adrenergic receptor-stimulated increase in cAMP in rat heart cells is not coupled to changes in Ca^{2+} dynamics, contractility, or phospholamban phosphorylation. *The Journal of Biological Chemistry*, 269(29), 19151–19156. <http://www.ncbi.nlm.nih.gov/pubmed/8034672>
- Xiao, R. P., & Lakatta, E. G. (1993). Beta 1-adrenoceptor stimulation and beta 2-adrenoceptor stimulation differ in their effects on contraction, cytosolic Ca^{2+} , and Ca^{2+} current in single rat ventricular cells. *Circulation Research*, 73(2), 286–300. <https://doi.org/10.1161/01.RES.73.2.286>
- Xu, C., Police, S., Rao, N., & Carpenter, M. K. (2002). Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circulation Research*, 91(6), 501–508. <https://doi.org/10.1161/01.RES.0000035254.80718.91>
- Yan, L., Jia, Z., Cui, J., Yang, H., Yang, H., Zhang, Y., & Zhou, C. (2011). Beta-adrenergic signals regulate cardiac differentiation of mouse embryonic stem cells via mitogen-activated protein kinase pathways. *Development, Growth & Differentiation*, 53(6), 772–779. <https://doi.org/10.1111/j.1440-169X.2011.01284.x>
- Yanagi, K., Takano, M., Narazaki, G., Uosaki, H., Hoshino, T., Ishii, T., Misaki, T., & Yamashita, J. K. (2007). Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels and T-Type Calcium Channels Confer Automaticity of Embryonic Stem Cell-Derived Cardiomyocytes. *Stem Cells*, 25(11), 2712–2719. <https://doi.org/10.1634/stemcells.2006-0388>
- Yang, X., Pabon, L., & Murry, C. E. (2014). Engineering adolescence: Maturation of human pluripotent stem cell-derived cardiomyocytes. In *Circulation Research* (Vol. 114, Issue 3, pp. 511–523). Circ Res. <https://doi.org/10.1161/CIRCRESAHA.114.300558>
- Yang, X., Rodriguez, M. L., Leonard, A., Sun, L., Fischer, K. A., Wang, Y., Ritterhoff, J., Zhao, L., Kolwicz, S. C., Pabon, L., Reinecke, H., Sniadecki, N. J., Tian, R., Ruohola-Baker, H., Xu, H., & Murry, C. E. (2019). Fatty Acids Enhance the Maturation of Cardiomyocytes Derived from Human Pluripotent Stem Cells. *Stem Cell Reports*, 13(4), 657–668. <https://doi.org/10.1016/j.stemcr.2019.08.013>
- Yang, X., Rodriguez, M., Pabon, L., Fischer, K. A., Reinecke, H., Regnier, M., Sniadecki, N. J., Ruohola-Baker, H., & Murry, C. E. (2014). Tri-iodo-L-thyronine promotes the maturation of human cardiomyocytes-derived from induced pluripotent stem cells. *Journal of Molecular and Cellular Cardiology*, 72, 296–304. <https://doi.org/10.1016/j.yjmcc.2014.04.005>
- Yazawa, M., Hsueh, B., Jia, X., Pasca, A. M., Bernstein, J. A., Hallmayer, J., & Dolmetsch, R. E. (2011). Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature*, 471(7337), 230–236. <https://doi.org/10.1038/nature09855>

- Yokoo, N., Baba, S., Kaichi, S., Niwa, A., Mima, T., Doi, H., Yamanaka, S., Nakahata, T., & Heike, T. (2009). The effects of cardioactive drugs on cardiomyocytes derived from human induced pluripotent stem cells. *Biochemical and Biophysical Research Communications*, 387(3), 482–488. <https://doi.org/10.1016/j.bbrc.2009.07.052>
- Yu, H., Xiong, S., Tay, C. Y., Leong, W. S., & Tan, L. P. (2012). A novel and simple microcontact printing technique for tacky, soft substrates and/or complex surfaces in soft tissue engineering. *Acta Biomaterialia*, 8(3), 1267–1272. <https://doi.org/10.1016/j.actbio.2011.09.006>
- Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, I. I., & Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science*, 318(5858), 1917–1920. <https://doi.org/10.1126/science.1151526>
- Zaccolo, M. (2006). Phosphodiesterases and compartmentalized cAMP signalling in the heart. In *European Journal of Cell Biology* (Vol. 85, Issue 7, pp. 693–697). Elsevier GmbH. <https://doi.org/10.1016/j.ejcb.2006.01.002>
- Zaccolo, M. (2011). Spatial control of cAMP signalling in health and disease. In *Current Opinion in Pharmacology* (Vol. 11, Issue 6, pp. 649–655). Elsevier Ltd. <https://doi.org/10.1016/j.coph.2011.09.014>
- Zaccolo, M., & Movsesian, M. A. (2007). cAMP and cGMP signaling cross-talk: Role of phosphodiesterases and implications for cardiac pathophysiology. In *Circulation Research*. <https://doi.org/10.1161/CIRCRESAHA.106.144501>
- Zaccolo, M., & Pozzan, T. (2002). Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science*, 295(5560), 1711–1715. <https://doi.org/10.1126/science.1069982>
- Zakhary, D. R., Moravec, C. S., & Bond, M. (2000). Regulation of PKA binding to AKAPs in the heart: alterations in human heart failure. *Circulation*, 101(12), 1459–1464. <https://doi.org/10.1161/01.cir.101.12.1459>
- Zhang, J., Wilson, G. F., Soerens, A. G., Koonce, C. H., Yu, J., Palecek, S. P., Thomson, J. A., & Kamp, T. J. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circulation Research*, 104(4), e30–41. <https://doi.org/10.1161/CIRCRESAHA.108.192237>
- Zhao, C. Y. (2017). *Computational Modeling of Cyclic Nucleotide Signaling Mechanisms in Cardiac Myocytes* (pp. 175–213). Springer, Cham. https://doi.org/10.1007/978-3-319-54579-0_10
- Zhao, C. Y., Greenstein, J. L., & Winslow, R. L. (2016). Roles of phosphodiesterases in the regulation of the cardiac cyclic nucleotide cross-talk signaling network. *Journal of Molecular and Cellular Cardiology*. <https://doi.org/10.1016/j.yjmcc.2016.01.004>
- Zhao, Z., Lan, H., El-Battrawy, I., Li, X., Buljubasic, F., Sattler, K., Yücel, G., Lang, S., Tiburcy, M., Zimmermann, W.-H., Cyganek, L., Utikal, J., Wieland, T., Borggrefe, M., Zhou, X.-B., & Akin, I. (2018). Ion Channel Expression and Characterization in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Stem Cells International*, 2018. <https://doi.org/10.1155/2018/6067096>
- Zhou, P., & Pu, W. T. (2016). Recounting cardiac cellular composition. In *Circulation Research* (Vol. 118, Issue 3, pp. 368–370). Lippincott Williams and Wilkins. <https://doi.org/10.1161/CIRCRESAHA.116.308139>

- Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K., & Xiao, R. P. (2001). Dual modulation of cell survival and cell death by β 2-adrenergic signaling in adult mouse cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 98(4), 1607–1612. <https://doi.org/10.1073/pnas.98.4.1607>
- Ziman, A. P., Gómez-Viquez, N. L., Bloch, R. J., & Lederer, W. J. (2010). Excitation-contraction coupling changes during postnatal cardiac development. *Journal of Molecular and Cellular Cardiology*, 48(2), 379–386. <https://doi.org/10.1016/j.yjmcc.2009.09.016>
- Zorn-Pauly, K., Schaffer, P., Pelzmann, B., Bernhart, E., Lang, P., Zink, M., Mächler, H., Rigler, B., & Koidl, B. (2003). A hyperpolarization activated inward current (I_f) is present in infant ventricular myocytes. *Basic Research in Cardiology*, 98(6), 362–366. <https://doi.org/10.1007/s00395-003-0424-9>
- Zwaveling, J., Winkler Prins, E. A., Pfaffendorf, M., & van Zwieten, P. A. (1996). The influence of hyperthyroidism on β -adrenoceptor-mediated relaxation of isolated small mesenteric arteries. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 353(4), 438–444. <https://doi.org/10.1007/BF00261441>
- Zwi, L., Caspi, O., Arbel, G., Huber, I., Gepstein, A., Park, I. H., & Gepstein, L. (2009). Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation*, 120(15), 1513–1523. <https://doi.org/10.1161/CIRCULATIONAHA.109.868885>