

FURTHERING THE DEVELOPMENT OF IN VITRO
MODELS IN CARDIOVASCULAR RESEARCH: FOCUS
ON A TITIN TRUNCATING VARIANT ASSOCIATED
WITH ATRIAL FIBRILLATION

by

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Declaration of own work and attribution

Unless otherwise stated, this thesis consists of my own work. Parts of Chapter 1 have been adapted from previously published work: Cumberland, M.J., Riebel, L.L., Roy, A., O'Shea, C., Holmes, A.P., Denning, C., Kirchhof, P., Rodriguez, B. and Gehmlich, K., 2022. Basic research approaches to evaluate cardiac arrhythmia in heart failure and beyond. *Frontiers in Physiology*, 13, p.806366. Chapter 3 is previously published work: Cumberland, M.J., Euchner, J., Azad, A.J., TN Vo, N., Kirchhof, P., Holmes, A.P., Denning, C. and Gehmlich, K., 2023. Generation of a human iPSC-derived cardiomyocyte/fibroblast engineered heart tissue model. *F1000Research*, 12, p.1224. Dr Naeromit Sontayananon (University of Oxford) performed the CRISPR-Cas9 engineering of the TTNtv iPSC line used in Chapter 4. Dr Albert Dasí (University of Oxford) and Ms Leto Riebel (University of Oxford) performed the computational modelling of the TTNtv in Chapter 4. Mr Jonas Euchner (University of Birmingham) performed the analysis on EHT contractility in Chapters 3 and 4. Dr Jinglei Yu (University of Birmingham) performed the quantitative proteomics runs in Chapter 5.

ABSTRACT

Background: Induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs) have revolutionised cardiac disease modelling over the past decade and are causing an inexorable shift towards the use of *in vitro* models. However, the utility of iPSC-CMs is still limited by the immaturity of the cells derived and their inability to recapitulate the complexities of cardiac disease. Co-culture 3D cardiac models confer tangible improvements in cell maturity and enable researchers to investigate the heterocellular cell to cell and cell to matrix interactions integral to the pathophysiology of heart disease.

Atrial fibrillation (AF) is a supraventricular tachyarrhythmia characterised by the rapid and irregular beating of the upper chambers of the heart. Dilated cardiomyopathy (DCM) is a type of heart disease that causes inefficient pumping function, which leads to heart failure.

Pathogenic variants that cause a truncation in the gigantic protein titin (TTNtv) are a genetic risk factor for the development of AF and DCM. The mechanism by which TTNtv can cause AF is currently obscure. The TTNtv, c.59926 + 1G > A, was previously identified as being causative in the development of DCM and AF.

Aim: Generate and validate atrial iPSC-CMs for compartment specific markers (Chapter 2). Develop an atrial 3D co-culture cardiac model using iPSC derived cardiomyocytes and cardiac fibroblasts (iPSC-CFs) (Chapter 3). Model the effect an AF predisposing TTNtv has on electrical and structural remodelling *in vitro* (Chapter 4). Investigate the utility of iPSC-CFs in exploring the pathophysiology of cardiac fibrosis (Chapter 5).

Methods: Atrial iPSC-CMs were generated and assessed for transcriptional, structural, and electrophysiological markers of compartment specificity. iPSC-CFs were differentiated, validated and incorporated into a 3D co-culture cardiac model. The c.59926 + 1G > A variant was engineered into iPSCs using CRISPR-Cas9 gene editing. The effect the variant had on

electrical and structural remodelling was assessed *in vitro* using patch clamp analysis and molecular biology techniques.

Results: The differentiated atrial iPSC-CMs demonstrated compartment specific transcriptional, structural and electrophysiological characteristics (Chapter 2). Addition of iPSC-CFs into a 3D co-culture model improved tissue compaction, contractile function and viability (Chapter 3). Consistent with previous *in vivo* studies, the TTNtv modelled increased the propensity for atrial fibrosis. Disruption to cholinergic stimulation in TTNtv atrial cardiomyocytes was observed *in vitro* and proved pro-arrhythmic when modelled *in silico* (Chapter 4). iPSC-CFs offered a more practical model than primary cardiac fibroblasts in which to assess cardiac fibrosis (Chapter 5).

Conclusions: This thesis evidences the utility of iPSC-CM/iPSC-CF 3D cardiac models in exploring the complexities of heart disease. For the first time, a TTNtv is shown to increase propensity for atrial fibrosis *in vitro*. Supporting previous evidence that TTNtv cause atrial remodelling and acting as valid mechanism for AF. Dysregulated cholinergic stimulation of cardiomyocytes is caused by TTNtv and represents a novel pathomechanism for arrhythmia.

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ABBREVIATIONS

ACFs	Atrial Cardiac Fibroblasts
AF	Atrial Fibrillation
AP	Action Potential
BMP-4	Bone Morphogenic Protein-4
CCh	Carbachol
cDNA	Complementary DNA
Ct	Cycle Threshold
CRISP/Cas9	Clustered Regularly Interspace Short Palindromic Repeat-Associated Protein 9
CVD	Cardiovascular Disease
DAPI	4',6-Diamidino-2-Phenylindole
DCM	Dilated Cardiomyopathy
DEGs	Differential Gene Expression
ECM	Extracellular Matrix
EHT	Engineered Heart Tissue
ELISA	Enzyme-Linked Immunosorbent Assay
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Het	Heterozygous
F-actin	Filamentous Actin
FDR	False Discovery Rate
FGF2	Fibroblast Growth Factor-2
FN1	Fibronectin 1
FN3	Type III Fibronectin
Ig	Immunoglobulin

I _K Ach	Acetylcholine-Dependent Inward-Rectifier Current
iPSC	Induced Pluripotent Stem Cell
iPSC-CFs	Induced Pluripotent Stem Cell Derived Cardiac Fibroblasts
iPSC-CMs	Induced Pluripotent Stem Cell Derived Cardiomyocytes
MLC-2a	Atrial Myosin Light Chain-2
MLC-2v	Ventricular Myosin Light Chain-2
mRNA	Messenger Ribonucleic Acid
PCA	Principal Component Analysis
PDGF	Platelet Derived Growth Factor
qPCR	Quantitative Polymerase Chain Reaction
RA	Retinoic Acid
SD	Standard Deviation
SEM	Standard Error Of Mean
T-Cap	Telethonin
TGF- β 1	Transforming Growth Factor- β 1
TRP	Transient Receptor Protein
TTN	Titin
TTNtv	Titin Truncating Variant
VCFs	Ventricular Cardiac Fibroblasts
WT	Wild-Type
Z-disc	Zwischenscheibe disc
α SMA	α -Smooth Muscle Actin

Chapter 1: INTRODUCTION

1.1 The Heart

The heart is the organ that is responsible for pumping blood around the 60,000 miles of vessels that supply the human body. It is an electrical pump that consists of 4 chambers: the upper right and left atria and the lower right and left ventricles (Wilcken, 2010). Contraction and relaxation of the chambers of the heart are respectively known by the terms systole and diastole. Contraction of the right and left atria (atrial systole) can help pump blood into the right and left adjoining ventricles (ventricular diastole). Contraction of the right and left ventricles takes places concurrently and is known as ventricular systole. The right ventricle pumps blood through the pulmonary artery to the lungs for reoxygenation and subsequently to the left atrium (atrial diastole). Contraction of the left ventricle pumps oxygenated blood through the aorta to the rest of the body and ultimately to the right atrium. The cardiac cycle is illustrated in *Figure 1.1*. Efficient and precise timing in the contraction and relaxation of the upper and lower chambers of the heart is mediated through the cardiac electrical conduction system.

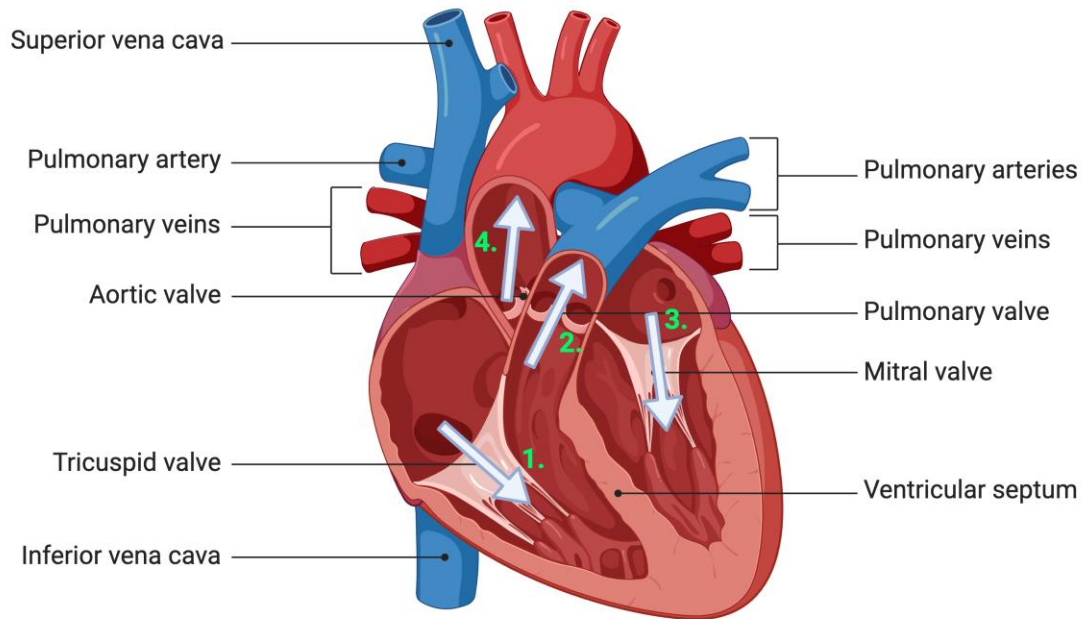


Figure 1.1: The Cardiac Cycle

The deoxygenated blood arrives in the right atrium through the inferior and superior vena cava. It is pumped through the tricuspid valve into the right ventricle (1). The blood passes through the pulmonary arteries (2) to the lungs where it undergoes oxygenation. It is carried into the left atrium from the pulmonary veins. It crosses the mitral valve (3) into the left ventricle where it is subsequently transported through the aorta (4) and to the rest of the body. Adapted from “Heart Anatomy”, by Berry, J. (2020) BioRender.com.

1.2 Cardiac Conduction System

The cardiac conduction system propagates electrical signals from the sinoatrial node, which acts as the pacemaker of the heart, to facilitate the coordinated contraction of the upper and lower chambers of the heart (Anderson et al., 2009). Gap junctions within the intercalated discs that connect adjacent cardiac muscle cells enable rapid exchange of charged ions and therefore the propagation of electrical signal (Rohr, 2004). These organelles are unique to cardiac muscle and allows it to operate as a functional syncytium, meaning signals can propagate to adjacent muscle cells in every direction. An outline of the cardiac conduction system is illustrated in *Figure 1.2*. A rapid change in membrane voltage, known as an action

potential, is generated by sinoatrial nodal cells in the upper right atrium. The signal spreads through the right atrium and into the left atrium via the Bachmann's bundle. Action potentials stimulate atrial cardiac muscle to contract, increasing pressure and ejecting blood into the lower chambers of the heart. The signal transmits to the atrioventricular node, which impedes signal propagation to prevent the atria and ventricles contracting at the same time. The signal travels down the bundle of His which bisects in the interventricular septum into the left and right bundle. The left and right bundle carry the signal to the Purkinje fibers, which activate and stimulate contraction of the cardiomyocytes in the left and right ventricles.

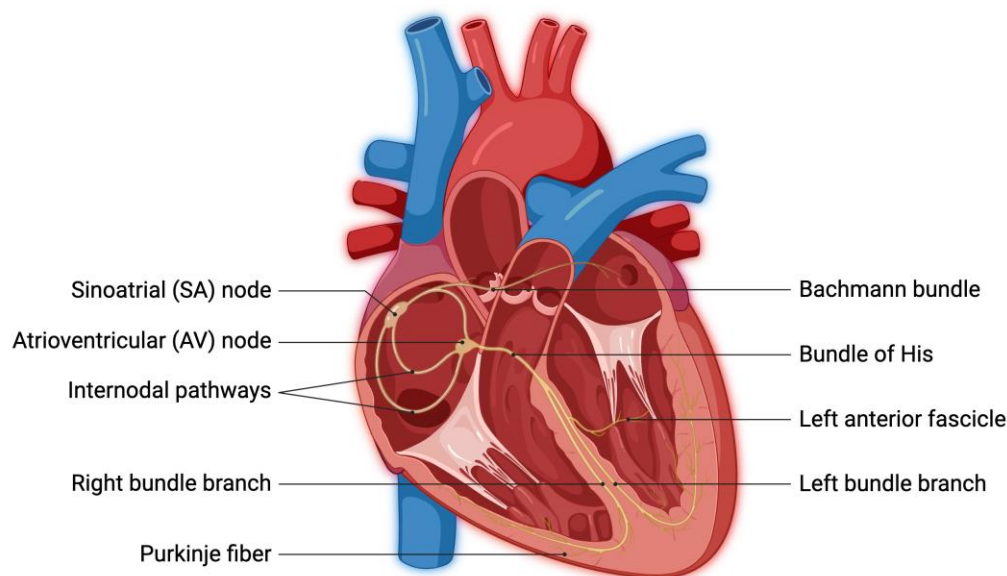


Figure 1.2: The Cardiac Conduction System

The signal spreads from the sinoatrial node to the right atrium and left atrium respectively via the internodal pathways and Bachmann bundle. This triggers contraction of the upper chambers of the heart. Following a 0.1 second delay at the atrioventricular node, the signal propagates through the Bundle of His to the Purkinje fibers, which activate and stimulate contraction of the ventricles. Adapted from "The Conducting System of the Heart", BioRender.com.

1.2.1 Electrocardiography

The electrical changes that occur during the cardiac cycle can be plotted in a voltage versus time graph, commonly known as an ECG (Geselowitz, 1989). Recognisable complexes within the ECG, such as the P wave, QRS complex and T wave, correspond to the depolarisation of the atria (P) and ventricles (QRS) and the repolarisation of the latter (T). Willem Einthoven is credited with the invention of electrocardiography and the contemporary ECG (Barold, 2003). Historically, the use of electrocardiography was integral in defining many of the fundamental mechanisms behind clinically important arrhythmias (Fye, 1994). Today the technique underpins a significant proportion of modern cardiovascular research and is extensively used to characterise electrical function in the hearts of patients and animal models. Cardiomyocytes are the cell type responsible for signal propagation in the heart.

1.3 Cardiomyocytes

The cardiac muscle cell or cardiomyocyte is one of the most populous cell types found in the heart (Litviňuková et al., 2020). Cardiomyocytes are rod-shaped muscle cells whose main function is to contract and relax upon electrical stimulation in order to generate the force needed to expel blood from a chamber of the heart. Cardiomyocytes found in different regions of the heart show slight differences in cellular physiology, structure and function. Cardiomyocytes can be broadly divided into force generating atrial and ventricular cardiomyocytes and pacemaker cells, which populate parts of the cardiac conduction system including the sinoatrial node, atrioventricular node and bundles of His. Pacemaker cells demonstrate automaticity, which is the intrinsic ability to independently instigate an action potential (Maltsev et al., 2007). This is achieved through the presence of a slow depolarizing current, known as the funny current (I_f), which is permeable to sodium (Na^+) and potassium (K^+) ions and is indirectly regulated by the autonomic nervous system (DiFrancesco and Borer, 2007).

1.4 Cardiac Action Potential

1.4.1 Phases of the Atrial and Ventricular Cardiac Action Potential

Atrial and ventricular cardiomyocytes have a highly negative 'hyperpolarised' resting membrane potential. This is primarily due to a high permeability to K^+ caused by increased levels of $K_{ir2.1}$ channels (I_{K1}). Additionally, the sodium-potassium pump helps maintain Na^+ and K^+ concentration gradients across the cell membrane by removing three Na^+ from the cell for the entry of two K^+ (Glitsch, 2001). Under healthy conditions, the duration of the action potential helps to determine the shortest time interval needed prior to a new stimulus. This is known as the effective refractory period. The action potentials of non-pacemaker cardiomyocytes can be broken down into the 4 phases shown in *Figure 1.3*. An action potential in a neighbouring cell causes a slight depolarisation of the cardiomyocyte from the resting membrane potential (**Phase 4**), as positive charge is transferred across the membrane through gap junctions. When the localised membrane potential reaches threshold, voltage-gated sodium channels (mainly $Na_v1.5$) are activated and there is a large influx of Na^+ into the cell (**Phase 0**) (Joukar, 2021). At approximately + 50 mV these sodium channels begin to inactivate, and the transient outward potassium current (I_{to}) causes brief but rapid K^+ efflux and partial repolarisation. A small influx of Cl^- also contributes towards rapid period of repolarisation. (**Phase 1**). Then, the membrane potential remains fairly constant, as there is a balance between K^+ efflux (caused by delayed rectifier potassium currents (I_{Kur} , I_{Kr} and I_{Ks})) and Ca^{2+} influx (caused by L-type calcium channels ($I_{Ca,L}$)) (**Phase 2**). The Ca^{2+} influx promotes release of Ca^{2+} from the sarcoplasmic reticulum (calcium-induced calcium release) which transfers to troponin C in the myofilament to elicit contraction. L-type Ca^{2+} channels inactivate and delayed rectifying potassium currents (I_{Kur} , I_{Kr} and I_{Ks}) dominate along with an increase in inwardly rectifying K^+ current (I_{K1}) such that the membrane potential repolarises

back towards the resting membrane potential (**Phase 3**). Action potentials can be recorded *in vitro* from cardiomyocytes using a variety of techniques including patch clamping.

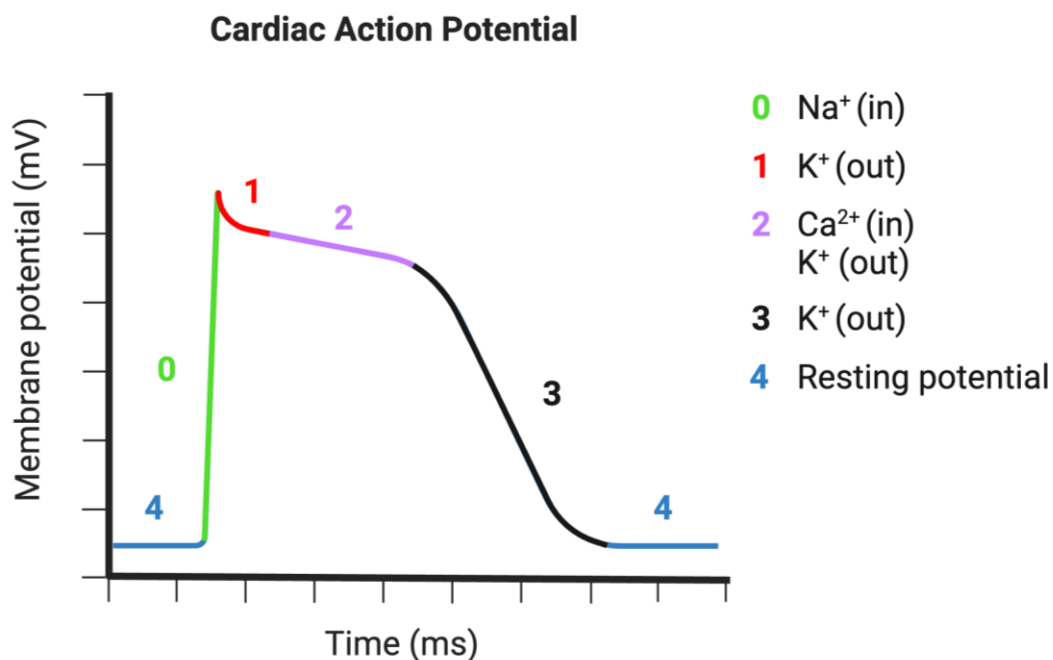


Figure 1.3: Cardiac Action Potential

The resting membrane potential is maintained at approximately -80 mV by IK_1 and the sodium potassium pump (4). Depolarisation of the membrane potential is caused by the rapid opening of sodium channels (0). The sodium channels close and the transient outward potassium current causes efflux of K^+ (1). The membrane potential remains relatively stable due to a balance in activity of L-type calcium channels and delayed rectifier potassium channels. Calcium influx causes calcium-induced calcium release from the sarcoplasmic reticulum and the cell contracts (2). The rapid delayed rectifier K^+ current and inwardly rectifying K^+ current cause membrane repolarisation (3). Created using Biorender.com.

1.4.2 Patch Clamping

Patch clamping is a technique used to study ionic currents and membrane potential in cardiac tissue samples and isolated cells. Patch clamping has and continues to be the gold standard for studying ion channel activity in excitable cells including cardiomyocytes and neurones (Guinamard et al., 2004; Alloui et al., 2006). There are a myriad of patch clamping setups used to monitor the electrophysiology of cells under a variety of controlled conditions. Many or which are outlined in detail in Kornreich, (2007).

Patch clamping can be broadly separated into two types. Voltage clamping involves “clamping” cardiac myocytes at different defined membrane potentials, to elicit and record

the function of specific currents. This often takes place in the presence of numerous pharmacological agents, which block other ion channels allowing for the isolation of a single current. Conversely, in the current clamp setup, the researcher ‘clamps’ the current flow across the cell membrane and records the membrane potential required to sustain this. This allows for determination of action potential morphology. Both setups are routinely used to study the impact that genetic variants, drug treatment, co-morbidities and environmental stressors have on ionic currents and action potential morphology in cardiomyocytes isolated from the upper or lower chambers of the heart (Chavali et al., 2019; Plant et al., 2020).

1.4.3 Atrial Versus Ventricular Action Potential

Ventricular and atrial action potentials differ in morphology and duration due to differential expression of key voltage gated ion channels. Contrasts in chamber specific intracellular electrophysiology contribute towards efficient cardiac conduction. The differences between the atrial and ventricular cardiomyocyte action potential are outlined in *Figure 1.4*. The resting potential of atrial cardiomyocytes (-65 to -80 mV) is slightly more depolarised than ventricular cardiomyocytes (-80 to -90 mV) due to differences in I_{K1} density (Trautwein et al., 1962). A more negative resting membrane potential in ventricular cardiac myocytes causes greater availability of sodium channels despite there being a lower relative expression of *SCN5A* and $Na_v1.5$. Consequently, the peak AP depolarisation (upstroke velocity) and AP amplitude is enhanced in ventricular cardiomyocytes. This is the principal reason for the greater conduction velocity in the lower chambers of the heart (O'Brien et al., 2022). A more prominent phase 1 notch is present in the atrial action potential due to differential expression of fast ($I_{to,f}$) and slow ($I_{to,s}$) transient outward potassium channels in atrial and ventricular cardiomyocytes. A less pronounced plateau phase in the atrial cardiac myocytes is due to the atrial specific expression of $K_v1.5$ (I_{Kur}), despite a predicted elevation in L-type Ca^{2+} current I_{CaL} (Grandi et al., 2011). The I_{Kur} and acetylcholine-activated inward rectifier potassium

current ($I_{K_{ACh}}$) contribute towards a relatively greater repolarisation rate in atrial cardiac myocytes (Kay and Shepard, 2017).

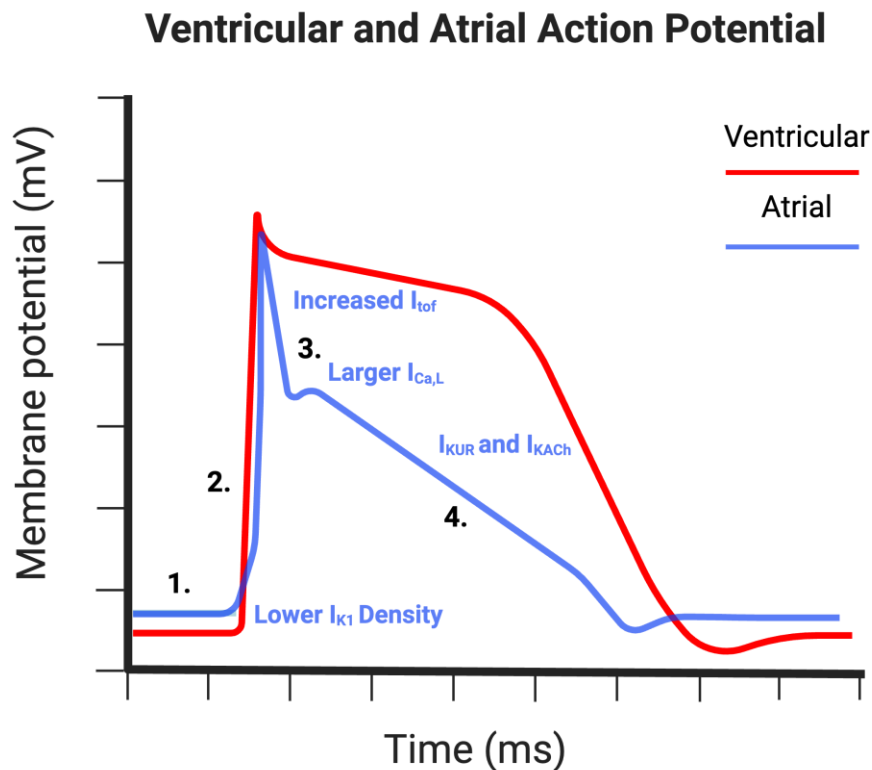


Figure 1.4: Atrial and Ventricular Cardiomyocyte Action Potential

Lower I_{K1} density in atrial cardiomyocytes causes a more positive membrane potential (1.). A more depolarised atrial membrane potential causes reduced sodium channel availability and leads to a slower upstroke velocity (2.). Increased I_{toF} causes a more pronounced notch in the atrial cardiomyocytes (3.). Greater L-type Ca^{2+} current contribution and the presence of I_{Kur} and $I_{K_{ACh}}$, lead to enhanced phase 3 repolarisation and an overall shortened action potential duration in atrial cardiomyocytes (4.). Created using Biorender.com.

1.4.4 Acetylcholine-activated potassium current ($I_{K_{ACh}}$)

The acetylcholine-activated potassium current ($I_{K_{ACh}}$) is an inwardly rectifying potassium current that is regulated by G-protein coupled receptors (GPCRs) rather than voltage gating (Jeevaratnam et al., 2018). Activation of $I_{K_{ACh}}$ increases outward K^+ conductance, leading to hyperpolarisation of the resting membrane potential and shortening of the APD. The $I_{K_{ACh}}$ is carried through channels consisting of heteromultimers of GIRK1 and GIRK 4 ($K_{ir}3.1$ and $K_{ir}3.4$) whose α -subunits are encoded for by the *KCNJ3* (GIRK1) and *KCNJ5* (GIRK4) genes respectively (András et al., 2021) (Corey and Clapham, 1998). The channel is predominantly

expressed in the atria, as well as the sinoatrial and atrioventricular nodes (Navarro-Polanco et al., 2013). (Krapivinsky et al., 1995). I_{KACH} is activated following vagal nerve stimulation of the heart. Post-ganglionic parasympathetic nerves originating in, or passing through, the ganglionated plexi release ACh which binds to membrane bound GPCR called muscarinic acetylcholine receptors (mAChR) (Kurachi, 1995). Upon receptor stimulation, the α subunit of the heterotrimer G protein interacts with the GIRK channel complex, causing I_{KACH} channel opening and the movement of K^+ out of the cell.

Pathological dysregulation of I_{KACH} can promote the development of a substrate capable of sustaining arrhythmias such as atrial fibrillation (AF) (Kneller et al., 2002). This is due to spatially heterogeneous reductions in the APD, which can promote re-entrant circuits (see section 1.8.3.2.1) (Nattel and Harada, 2014). Furthermore, chronic AF is associated with an agonist independent constitutive form of I_{KACH} which can facilitate the maintenance of AF via enhanced APD shortening and hyperpolarisation of the atrial RMP (Cha et al., 2006). Rapid supraventricular rates caused by AF can lead to atrial tachycardia induced remodelling of the atria. Atrial tachycardia induced remodelling causes a switch to this constitutive form of I_{KACH} , that further promotes the development of AF (AF begets AF, see section 1.8.3.2) (Wijffels et al., 1995). Activation of the chronic form of I_{KACH} is caused by differential protein kinase C isoform regulation (Makary et al., 2011) (Chang et al., 2022). It is currently unknown if changes in I_{KACH} contribute to the increased AF incidence in patients with TTNtv. It will therefore be evaluated in this current thesis.

1.5 Cardiac Sarcomere

The cardiac sarcomere is the smallest repeated unit of a cardiomyocyte and is responsible for contraction and force generation. Sarcomeres are composed of 3 myofilaments: the thick, thin and elastic (de Tombe, 2003). Sarcomeres are found inside the sarcolemma of cardiomyocytes, wrapped in a membranous organelle responsible for calcium handling,

known as the sarcoplasmic reticulum (Martin and Kirk, 2020). Sarcomeres are arranged into rod like myofibrils which lie in parallel to form a myofiber. Cardiac myofibers are found in the myocardium of the heart and work together to develop the pressure which drives blood out of a cardiac chamber. The thick myofilament contains motor protein myosin II, which has actin binding myosin heads protruding at an angle from either side of the molecule (Crocini and Gotthardt, 2021). The thin and elastic filaments contain F-actin and titin respectively. The sarcomere is defined as the area between 2 adjacent 'zwischen' (German meaning of 'between') or Z-lines. The I-band is located directly next to the Z-line and contains regions of the thin and elastic filaments (Martin and Kirk, 2020). The A-band appears as a denser region under a light microscope and is composed of thick, thin, and elastic filaments. The M-line or middle line bisects the sarcomere and acts as an anchor for the thick filaments. Titin molecules traverse half the length of the sarcomere from the Z-line to the M-line, forming the elastic filament. The thin filament is anchored to the Z-line by the crosslinking protein α -actinin (de Tombe, 2003). A diagram of a contracted and relaxed sarcomere is illustrated in *Figure 1.5*. During muscle contraction, the release of Ca^{2+} from the sarcoplasmic reticulum enables the myosin heads of the thick filaments to bind to the thin filaments and pull them inwardly towards each other. This causes the thick and thin filaments to overlap, shortening the length of the sarcomere and generating force. The elastic filament, composed of the gigantic protein titin, helps regulate function, structure and signalling within the sarcomere.

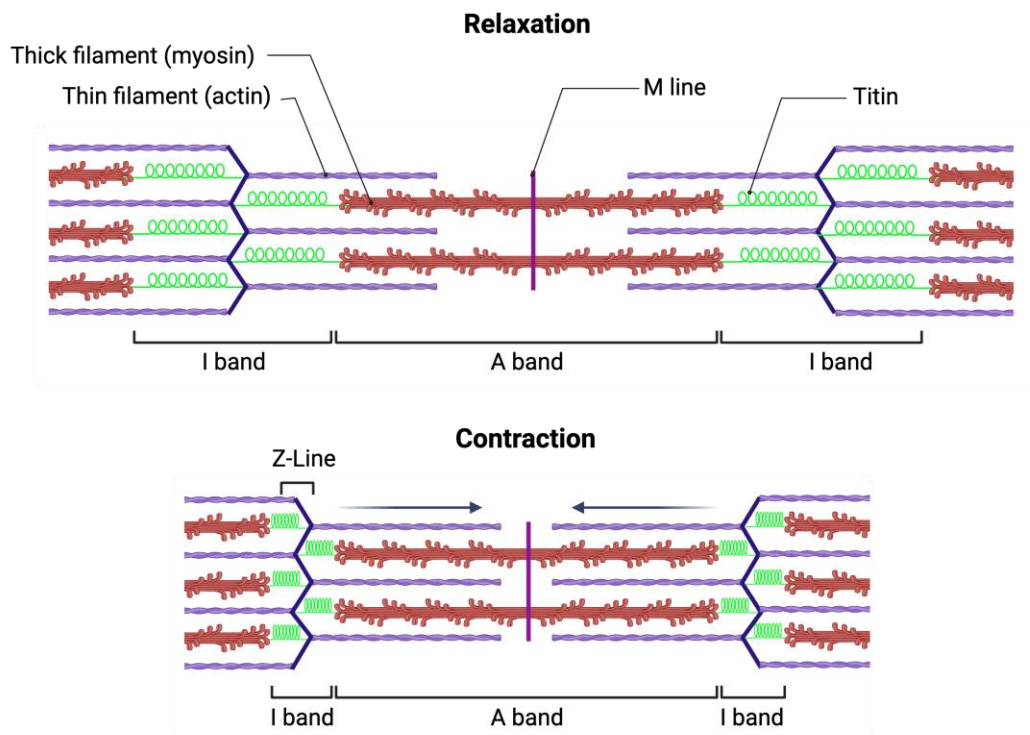


Figure 1.5: The Sarcomere

The sarcomere contains 3 myofilaments: thick, thin, and elastic. During contraction, the myosin heads of the thick filaments bind to thin filaments and pull them inwards, towards the M-line. This causes the thick and thin filaments to overlap and shortens the sarcomere. Adapted from 'Sarcomere Contraction' Martini, (2003) Biorender.com.

1.6. Titin

1.6.1 Overview of Titin

In humans the gigantic protein titin is encoded by the *TTN* gene. The gene is found on the long arm of chromosome 2 (2q31), consists of 363 exons and encodes approximately 35,000 amino acids (Bang et al., 2001). Extensive alternative splicing means that a number of isoforms of the protein have been detected, with the largest having a molecular weight of approximately 4 MDa. This makes titin the largest known natural protein. Titin is the third most abundant protein in striated muscle, after the contractile proteins myosin and actin (Labeit et al., 1997). It has a multifunctional role in the sarcomeres of striated muscle myofibrils, contributing towards structure, function, and signalling (Granzier and Labeit,

2007). In striated muscle titin acts as a molecular spring. It generates passive tension within the muscle and helps to regulate the length of the sarcomere prior to and post contraction. Titin is also thought to contribute to the assembly of the sarcomere, acting as molecular scaffolding for the construction and arrangement of the contractile myofilaments (Zou et al., 2006). Titin plays an important role in hypertrophic signalling processes and sarcomeric protein turnover (Kötter et al, 2014). The structure of titin is complex but integral to its function.

1.6.2 Domain Organisation of Titin

Titin is made up of approximately 300 separately folded protein domains. The majority of titin is composed of two types of protein domains, immunoglobulin (Ig) and type III fibronectin (FN3) (Tskhovrebova, and Trinick, 2003). The structured protein domains consist of β -sandwich folds of seven or eight strands and are linked together by unstructured peptide sequences. Most titin isoforms are over 1 μm in length and traverse half the length of the sarcomere from the Z-disc to the M-line. The domain structure of titin is shown in *Figure 1.6*. The region of titin that traverses the I-band of the sarcomere is composed of a N2 region flanked by immunoglobulin domains (Ig). The N2 region is adjacent to a segment rich in the amino acids proline, glutamate, valine and lysine, appositely known as the PEVK sequence. The I-band region of titin connects the thick myosin filament to the Z-disc, maintaining its central orientation in the sarcomere following contraction and extension (Horowitz et al., 1986). The region of titin that traverses the A-band is highly conserved. It is made up of Ig and FN3 domains arranged in long sequences known as super repeats (Amodeo et al., 2003). The A-band of titin interacts with myosin and runs parallel with the thick filament to the M-line of the sarcomere. The A-band region of titin is thought to determine the length of the thick filament during sarcomere assembly (Van der Ven et al., 2000). The M-line region of

titin contains binding sites for important signalling proteins and a serine/threonine kinase domain. (Peng et al., 2007). It has putative structural and signalling functions.

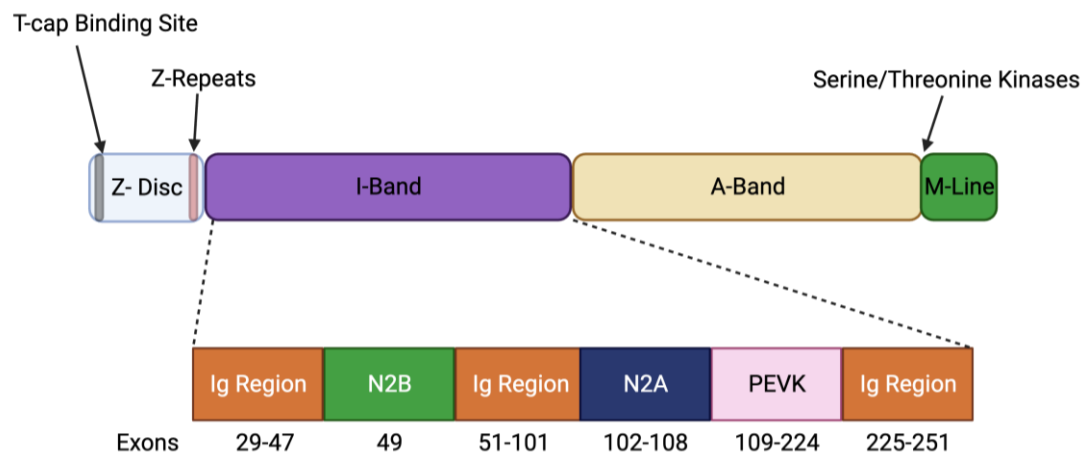


Figure 1.6: Domain Structure of Titin

The Z-disc is composed of Ig domains and has a binding site for the signalling protein telethonin (T-Cap). The Z-repeats are located adjacent to the T-cap binding site and act as a crosslinking site for α -actinin and the thin filaments. Regions of the I-band are shown below, with the N2B, N2A and PEVK regions acting as elastic elements to generate passive force during relaxation. The A-band contains binding sites for myosin and myosin binding protein C and is composed of super repeats that correspond to helical repeats in the thick filament. The M-line is made up of Ig domains interspersed with unique sequences. A serine/threonine kinase located between the A-Band and the M-Line is important in myofibrillogenesis. The M-line contains binding sites for myomesin and M-protein to enable tethering of the thick filament to the M-line. Created using Biorender.com.

1.6.3 Structure and Function of Titin

1.6.3.1 Z-Disc titin

The N-terminal region of titin is located at the Z-disc of the sarcomere, where it overlaps with the titin of the adjacent sarcomere. The Z-disc region is made up of Ig domains interconnected by unique sequences. The N-terminus of titin has a binding site that facilitates strong binding of telethonin (T-cap), a signalling protein that has a functional role in sarcomere turnover and assembly (Mues et al., 1998). Distal to the T-cap binding domain is a unique sequence containing 45 residue repeats, known as Z repeats. α -actinin binds to the Z-repeats to function as the connector in a titin- α -actinin- actin complex. Overlapping N-terminals of titin in the Z-disc means that the thin myofilaments of adjacent sarcomeres are linked together. This linkage fastens titin to the Z-disc and helps to stabilize the ultrastructure

of the sarcomere (Young et al., 1998). Two immunoglobulin domains proximal to the Z-repeats act as a binding site for obscurin, a signalling protein that contributes to the assembly of sarcomeres (Borisov et al., 2004).

1.6.3.2 I-Band Region titin

The N and C-terminals of the I-band region of titin respectively contain tandem arrays of 15 and 22 constitutively expressed Ig domains. The domains are bifurcated by a variable sequence of extensible Ig domains, the aforementioned N2 region and PEVK spring. The sequence of the N2 region varies in skeletal and cardiac muscle. Skeletal muscle cells express an isoform of titin with only a N2A region, while the N2B and N2BA (N2B+N2A) isoforms are found in cardiomyocytes (*Figure 1.6*) (Nagueh et al., 2004). The extensible elements of the I-band region behave like a molecular spring, extending with the sarcomere during muscle relaxation and recoiling during contraction to generate passive tension within the muscle. Variability of the I-band region contributes to the difference in elasticity of muscle types. This is of particular importance in the heart, where the additional hyper-elastic N2B motif helps to dictate the passive stiffness of the sarcomeres during diastole (Elhamine et al., 2014). The I-band of titin also has a role in signalling. Components of the elastic spring can be post-translationally modified to alter the stiffness of sarcomeres (Krüger et al., 2009). The N2A element also contains a binding site for the important but somewhat enigmatic protease calpain-3, which has speculative roles in sarcomeric repair and turnover (Ojima et al., 2008) (Beckmann and Spencer, 2008).

1.6.3.3 A-Band Region titin

The super repeats of the A-band region can be composed of 7 or 11 Ig and FN3 domains. The distance spanned by a 11 domained super repeat corresponds to that of the myosin helical repeat (Furst et al., 1989). This has led to the so called “molecular ruler hypothesis”. A hypothesis that is still controversially discussed and proposes a functional role of the A-band

of titin in stipulating the length of the thick filament in the sarcomere. Binding sites for myosin and myosin binding protein C in the A-band region enable titin to act as a hub for the formation of an actomyosin complex, supporting the molecular ruler hypothesis (Freiburg and Gautel, 1996) (Muhle-Goll et al., 2001). However, studies have shown that deletion of large segments of the I/A band junction cause no significant change in thick filament length in mouse models (Granzier et al., 2014). This indicates that the role the A-band has in determining the length of the thick filament is more intricate than the “molecular ruler hypothesis” suggests and requires further research to elucidate.

1.6.3.4 M-Line titin

The portion of titin located inside the M-line of the sarcomere is made up of Ig and FN3 domains concatenated by unique sequences. It is predominantly involved in myocyte signalling and has shown to be important to sarcomere assembly and turnover (Musa et al., 2006). A serine/threonine kinase domain, located at the A-band/M-line junction of titin, can be activated by the calcium-binding messenger protein calmodulin during myofibrillogenesis (Mayans et al., 1998). This can then in turn help upregulate hypertrophic genes via MURF2 activation (Lange et al., 2005). The myosin binding proteins myomesin and M-protein bind to titin at sites located in the M-line (Van Der Ven and Fürst, 1997). Myomesin and M-protein tether thick filaments to the M-line, providing structural support and function analogous to that played by α -actinin at the Z-line (Agarkova and Perriard, 2005). A kinase domain located towards the carboxy-terminus of titin is thought to have mechano-regulatory functions in sarcomeric protein turnover and tension control. The aforementioned Z-disc binding protein telethonin has been identified as a potential substrate for the kinase domain of titin. This further implicates titin as being a key regulator of sarcomere assembly (Mayans et al., 1998). The substrates and function of this kinase domain are still obscure. A myriad of pathogenic

variants have been identified in the *TTN* gene that disrupt its structure to cause heritable muscle disease.

1.7 Titin in Human Disease

1.7.1 Titin and Myopathy

Linkage analysis first implicated pathogenic variants of titin in the skeletal myopathy, autosomal recessive nemaline myopathy, in 1997 (Pelin et al., 1997). Variants near the binding sites of obscurin and calpain-3 in the I-band region of titin were shown to be clinically significant in the skeletal diseases, tibial muscular dystrophy and limb-girdle muscular dystrophy type 2J (Haravuori et al., 2001) (Hackman et al., 2002) (Sarparanta et al., 2010). The expansiveness of the gene limited our early understanding on the pathogenicity of its variants. Following the invention of next-generation sequencing technologies, it became feasible to sequence the entire gene in large cohorts of individuals. Pathogenic variants of titin have since been implicated in centronuclear and multi-minicore myopathies, as well as hereditary cardiovascular diseases including atrial fibrillation (AF) and cardiomyopathy (Savarese et al., 2016).

1.7.2 Cardiovascular Disease

Cardiovascular disease (CVD) remains the leading cause of death globally and is responsible for approximately 20 million deaths a year (Gaidai et al., 2023) (Harikrishnan et al., 2020). Cardiovascular disease encompasses a wide array of diseases relating to the heart and vasculature. Types of cardiovascular disease include stroke, coronary heart disease and heart failure (Gaziano et al., 2006). The diverse number of cardiovascular diseases means that there is no one common aetiology. Smoking, obesity and hypertension are however known risk factors that predispose individuals to a broad range of cardiovascular diseases (Mozaffarian et al., 2008). Heart disease is a term that relates specifically to any disease that affects the heart and includes arrhythmia, valve disease and cardiomyopathy (Virani et al., 2020).

Cardiomyopathy is a general term used to describe a group of diseases that affect the cardiac muscle. Cardiomyopathy causes changes in the size and morphology of the heart that reduce its efficiency to pump blood, independently from risk factors (e.g. hypertension) that would explain such changes (Brieler et al., 2017).

1.7.3 Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) is a disease of the heart where the left ventricle becomes enlarged, and the normally muscular left ventricular wall becomes thin and weak. These changes can cause systolic dysfunction of the heart (Haas et al., 2015). Individuals with DCM can be asymptomatic or present a range of symptoms from shortness of breath and fatigue to severe cardiac arrhythmias. DCM affects approximately 1:250-2500 people and presents a significant financial burden to national health services (Merlo et al., 2014). This is due to the serious ramifications of the disease. The 5-year survival rate of patients diagnosed with DCM is approximately 50 %, this is due to the frequent development of congestive heart failure (Grogan et al., 1995). DCM is also the leading cause of paediatric heart transplantation (Kirk et al., 2008). The European Society of Cardiology (ESC) classifies DCM as being either familial (genetic) or non-familial (acquired) (Elliot et al., 2008) (Schultheiss et al., 2019). Non-familial dilated cardiomyopathy is associated with excessive alcohol use, pregnancy, tachycardia, myocarditis, hypertension and coronary artery disease (Luk et al., 2009). However, the primary driver in many cases is unclear.

Approximately half of all DCM cases are thought to be familial, with an inherited pathogenic variant causing the disease (McNally et al., 2013). The genetic cause can be identified in around 40 % of patients with familial DCM (Hershberger et al., 2013) (Ganesh et al., 2013). This means that a large proportion of DCM cases are still considered idiopathic. As with other inherited heart diseases, the pathogenesis of familial DCM is complex. Hemodynamic stress plays an important role in the manifestation of the disease in individuals with known

relevant mutations (Bondué et al., 2018). Pathogenic variants in several sarcomeric genes have been identified as some of the most highly linked to familial dilated cardiomyopathy including *FLNC*, *LMNA* and *MYH7* (Njoroge et al., 2022) (Fatkin et al., 2014). *TTN* variants are the largest genetic determinant of familial DCM (Herman et al., 2012).

1.7.4 Pathogenic Titin Variants and Dilated Cardiomyopathy

1.7.4.1 Titin Missense Variants

In 1999, pathogenic variants of titin were linked to the development of hypertrophic and dilated cardiomyopathy following the Sanger sequencing of specific sections of the gene in affected individuals (Satoh et al., 1999) (Siu et al., 1999). A missense variant is the non-synonymous substitution of one amino acid for another caused by a point mutation. *TTN* missense variants are associated with DCM, but the role they play in its development is ill-defined. *TTN* missense variants are found to be more prevalent in DCM patients than in the general population, however carrying a missense variant has been shown to cause no detrimental effect on DCM patient prognosis (Begay et al., 2015). A small subset of *TTN* missense variants, found in structurally integral regions of the gene, demonstrate markers of DCM causality. An example of a considerably penetrant missense variant is the Trp930Arg variant located at the Z-disc/I-band junction observed in large families affected by hereditary DCM (Gerull et al., 2002). A study by Domínguez et al., (2023) has recently confirmed experimentally that *TTN* missense variants can cause familial DCM.

1.7.4.2 Overview of Titin Truncating Variants

Variants in titin that are thought to truncate the length of the protein (frameshifts, nonsense and essential splice site mutations) are known as titin-truncating variants (TTNtv). They are found in approximately 1 in 5 individuals diagnosed with familial dilated cardiomyopathy, making them the most prevalent genetic cause of the disease (Herman et al., 2012) (Akinrinade et al., 2015). TTNtv have also been implicated as causative in arrhythmogenic

right ventricular and familial restrictive cardiomyopathy (Taylor et al., 2011) (Peled et al., 2014). As is the case in dilated cardiomyopathy, TTNtv are the most common predisposing variant in peripartum cardiomyopathy (Ware et al., 2016). Peripartum cardiomyopathy presents towards the end of pregnancy or within the first months following delivery (van Spaendonck-Zwarts et al., 2014). It is an idiopathic cardiomyopathy characterised by left ventricular dysfunction and heart failure. As with those linked to DCM, the TTNtv found in patients with peripartum DCM are localised to the A-band region of titin (Spracklen et al., 2021).

Most TTNtv found in DCM patients are heterozygous, i.e. they co-exist with a wild-type titin allele. Penetrance of TTNtv is unexpectedly low, with approximately 1-3 % of the population possessing a truncating variant (Akinrinade et al., 2015). This can be partly explained by the occurrence of TTNtvs in exons that are commonly spliced out in adult transcripts, or are located in regions unincorporated into cardiac isoforms, hence these TTNtv are predicted to be silent. However, it is also likely that environmental stressors play a role in the unshrouding of TTNtv and its subsequent pathophysiology. Substantial age-dependent penetrance is seen in TTNtv associated DCM and supports the occurrence of a gene-environment interaction (Franaszczyk et al., 2017). Environmental triggers such as viral infection, alcohol intake and autoimmune disease have shown to be causal in a significant proportion of idiopathic DCM cases and likely play a role in the manifestation of familial DCM (Martino et al., 1994) (Jahns et al., 2004) (Fabrizio and Regan, 1994). DCM patients with excess alcohol consumption have a significantly lower ejection fraction if they carry a TTNtv (Ware et al., 2018). This indicates that environmental stressors likely play a role in TTNtv DCM. Pathogenic TTNtv are predominantly found in constitutively expressed exons, such as those that encode regions of the A-band, but have also been detected in the Z-disc, I-band and M-line of titin (Herman et al., 2012) (Schafer et al., 2017). The severity of the TTNtv increases with proximity to the

C-terminal of titin. One proposed reason for this positional effect is that a longer, more “poisonous” peptide is formed when the truncation occurs closer to the C-terminus (Roberts et al., 2015). Another reason suggested in Zou et al., (2015) is that the effect is due to the variant being located downstream of an intragenic promoter called “cronos”. Variants upstream of the promoter are unlikely to affect the reading frame of cronos and can thus be “rescued” by the alternative isoform transcribed by the promoter. In Zaunbrecher et al., (2019), homozygous TTNtv were introduced into the Z-disc and A-band of *TTN* in stem cells using CRISPR/Cas9. Cardiomyocytes derived from the stem cells containing the A-band TTNtv, which was located downstream of the cronos promoter, displayed significant disruption to sarcomere and myofibril formation. This was thought to be due to cells expressing deleterious truncated isoforms of full-length and cronos titin. In contrast to this, the cardiomyocytes with the Z-disc mutation were thought to be rescued by the expression and subsequent incorporation of cronos titin into the sarcomeres. The role that the cronos isoform of titin has on the pathogenicity of TTNtv is still obscure, recent studies suggest that is unlikely to play a part in the pathomechanism (Fomin et al., 2021).

1.7.4.3 Pathomechanism of TTNtv associated Dilated Cardiomyopathy

The pathomechanism behind TTNtv associated DCM is currently unclear. There are currently three main mechanisms proposed: haploinsufficiency, translation of a “poisonous” polypeptide and disruption to cardiac signalling/metabolism. A brief explanation of these mechanisms is given below.

1.7.4.3.1 Haploinsufficiency

Difficulty in isolating truncated titin protein isoforms sparked the idea that haploinsufficiency is responsible for the pathomechanism of TTNtv (Gramlich et al., 2009). The mechanism proposed that nonsense mediated decay of atypical *TTN* transcript leaves the cell in a haploinsufficient state and that DCM may occur when compensating for these changes

(Tabish et al., 2017). Indeed, nonsense mediated decay of truncated *TTN* transcripts has been shown in mutant rat models, by Schafer et al., (2017). The group showed that despite wild-type titin being slightly upregulated to compensate for the heterozygous mutation, there was still 40 % less sarcomere spanning titin. Further to this, a conditional knock out of *TTN* was generated in a mouse model by Radke et al., (2019) to cause a gradual postnatal decrease in *TTN* expression. Sarcomere disassembly and striated muscle atrophy occurred with the progressive depletion of *TTN*, demonstrating the importance of gene expression. It is interesting to note that homozygous deletions in the M-line of *TTN* have been associated with severe early-onset dilated cardiomyopathy (Carmignac et al., 2007). This demonstrates that gene-dosage plays an important role in the pathogenesis of TTNtv, however it is not necessarily indicative of a haploinsufficiency based mechanism.

There is however evidence against a haploinsufficiency based mechanism. The effect that the location of the TTNtv has on the severity of the variant is broadly inconsistent with a haploinsufficiency based pathomechanism. If haploinsufficiency was the primary reason for TTNtv associated DCM then variants would be expected to have similar pathogenic effect independent of their location. Furthermore, recent detection of the truncated titin isoform by McAfee et al., (2021) and Fomin et al., (2021) in the sarcomeres of cardiomyocytes casts doubt that haploinsufficiency is the defining pathomechanism for DCM. Both groups suggest that haploinsufficiency plays a role in the pathomechanism of TTNtv DCM, but that sarcomeric titin insufficiency is caused by either reduced translation efficiency of the transcript or by increased degradation of the truncated protein. Neither group, identified differences in total *TTN* gene expression. A notion that has since been supported by Stroeks et al., (2023). It is worth noting that all three of these studies investigated *TTN* gene expression in the hearts of patients that had already been diagnosed with DCM. Reduced *TTN* gene expression may be crucial to the initial development of TTNtv associated DCM.

1.7.4.3.2 *Poison Peptide Hypothesis*

A dominant negative mutation is one where the translated product of the mutated allele affects the function of the wild type allele, within the same cell. It is colloquially known as the “poison peptide” mechanism when discussing the pathomechanism of TTNtv. A study performed in Roberts et al., (2015) on samples collected from TTNtv DCM patients supported a poison peptide based pathomechanism by showing a strong length dependent effect and no evidence of nonsense mediated decay. The detection of truncated titin isoforms in stem cell derived cardiomyocytes by Hinson et al., (2015) led the group to hypothesise that the incorporation of truncated isoforms into the sarcomere causes mechanical dysfunction. Indeed, the group found that sarcomerogenesis and consequently contractile force was impeded in the TTNtv cardiomyocytes. This was thought to be due to the loss of the β -cardiac myosin binding site in the truncated titin isoform. This may explain the position dependent severity of TTNtv, with mutations occurring towards the C-terminus of the A-band, being more likely to disrupt the β -cardiac myosin binding site. In contrast to this, TTNtv were shown to affect passive stiffness but not contractile force in primary cardiomyocytes isolated from TTNtv DCM patients (Vikhorev et al., 2017). Recent studies have detected the presence of truncated titin isoforms in cardiomyocytes isolated from patients with TTNtv DCM. Fomin et al., (2021) detected truncated titin proteins in intracellular aggregates outside of the sarcomeres of the cardiomyocytes which may disrupt protein quality control. McAfee et al., (2021) detected truncated isoforms of the protein in the heart but were unable to determine if they localised to the sarcomeres. Evidence that truncated titin isoforms are structurally integrated into the sarcomeres of cardiomyocytes has recently been illustrated using super resolution microscopy (Kellermayer et al., 2023). It was hypothesised to cause disease by disrupting mechanosensory function.

1.7.4.3.3 *Disruption to Cardiac Signalling and Metabolism*

Disruption to cardiac metabolic pathways has been implicated in TTNtv associated DCM.

The signalling complex mTORC1 is an important nutrient sensor with a key role in protein synthesis (Bar-Peled and Sabatini, 2014). Changes to nutrient availability within the heart can cause activation of mTORC1 (Jewell and Guan, 2013). This aberrant activation has been linked to maladaptive responses such as autophagy inhibition (Ramos et al., 2012).

Disruption to autophagy has been found in numerous cardiomyopathies, including DCM (Zech et al., 2020). In rats, truncations in the A band and Z-disc of titin cause a metabolic shift from fatty acid metabolism to glycolysis. The RNA-seq and Ribo-seq data obtained from the A-band and Z-disc mutants demonstrated position independent NMD of the mutant alleles and a similar reduction in full length titin synthesis (60 % of wild type). There was no significant difference between the respective transcriptome and translome of the mutants. A shift away from fatty acid metabolism and an increased reliance on glycolysis is often seen in failing hearts and was shown to cause a subsequent increase in metabolites that activate mTORC1 in the mutant rats (Schafer et al., 2017). Activation of mTORC1 in non-ischemic DCM patients was shown by Yano et al (2016). The increase in mTORC1 activity was linked to the subsequent progression of patients to heart failure. More recent studies have shown that TTNtv can cause significant changes in mitochondrial function in both humans and rats (Verdonschot et al., 2018) (Adams et al., 2019). It is presently unclear if the changes in mitochondrial function are linked to mTORC1 activation and if they are responsible for the pathomechanism of the disease or are merely a secondary consequence. Other studies have implicated changes in calcium handling, caused by disruption to SERCA and phospholamban, as being key to the pathomechanism of TTNtv DCM. Further study is required to conclude whether changes in cardiac metabolism are key to the pathology of TTNtv or are secondary effects (Schick et al., 2018).

1.8 Titin and Arrhythmia

1.8.1 Arrhythmia

An arrhythmia is a condition of the heart where the heart beats too fast (tachycardia), too slow (bradycardia) or in an irregular manner (fibrillation). Arrhythmias are often classified by the origin of the abnormal electrical impulse, with atrial and ventricular arrhythmias occurring respectively in the upper and lower chambers of the heart. Arrhythmias can be asymptomatic or can present with a range of symptoms including shortness of breath and fainting. Many arrhythmias are relatively benign; however, some can increase the risk of the individual to heart failure, ischemic stroke and sudden cardiac death (Olsson et al., 2006) (Pozzoli et al., 1998) (Luu et al., 1989). Ventricular tachycardia and fibrillation are two of the most common types of arrhythmia that can lead to cardiac arrest and sudden cardiac death (Neumar et al., 2015).

The most common type of arrhythmia, atrial fibrillation (AF), occurs when disorganised electrical impulses fire in the upper chambers of the heart. It can cause fast and irregular heartbeats. AF is present in approximately 3% of the population and was responsible for 115,000 deaths worldwide in 2013 (Friberg and Bergfeldt, 2013) (Abubakar et al., 2015). The treatment given to patients with arrhythmia is dependent on the type and severity of the condition. Anti-arrhythmic drugs such as fast-channel and beta-blockers can help ameliorate ventricular tachycardias and AF (Lei et al., 2018). Catheter ablation allows clinicians to localise and nullify the origin of the arrhythmia, by targeting the tissue with radiofrequency energy or by freezing the tissue (O'Neill et al., 2007). Pacemakers and implantable cardioverter defibrillators (ICD) are cardiac devices that help prevent or terminate arrhythmia in the heart by providing electrical stimulation (DeSilva et al., 1980) (Tchou et al., 1988). The development of cardiac arrhythmias is often associated with prior myocardial ischemia, cardiomyopathy and cardiac fibrosis (Janse and Wit, 1989) (Kiès et al., 2006) (Iles et al.,

2011). Cardiac arrhythmias can present concurrently in the development of some cardiomyopathies and can be causal in others e.g. tachycardia-induced dilated cardiomyopathy (Umana et al., 2003). It can therefore be difficult to differentiate between arrhythmias that occur prior or during the development of cardiomyopathy and those that extrinsically arise from cardiomyopathy induced heart remodelling. Familial arrhythmic syndromes are a group of inherited diseases that can causes arrhythmia in the absence of structural heart disease. Many of these diseases, including Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia and Long QT syndrome, are caused by pathogenic variants in ion channels and are eponymously known as channelopathies (Gray and Behr, 2016).

1.8.2 Arrhythmias in Dilated Cardiomyopathy

Approximately one-third of individuals with dilated cardiomyopathy present an arrhythmogenic phenotype during the initial stage of the disease (Spezzacatene et al., 2015). Arrhythmias are one of the earliest manifestations of DCM and have proven clinically important in their diagnosis (McNair et al., 2011) (Van Berlo et al., 2005). The presence of arrhythmic events at the early stages of the disease, increases the likelihood of sudden cardiac death in DCM patients (Meinertz et al., 1984). A literature analysis performed by Peters et al., (2019) highlighted the importance of 11 key genes associated with arrhythmias in familial DCM. Variants of the genes encoding the proteins laminin A/C and titin were among those that conferred the greatest propensity for atrial/ventricular arrhythmias in DCM.

1.8.3 Titin Truncating Variants and Arrhythmias

1.8.3.1 Arrhythmia in *TTN*tv Dilated Cardiomyopathy

DCM patients with *TTN*tv are more likely to develop atrial and ventricular arrhythmias during the early and late stages of the disease (Tayal et al., 2017) (Verdonschot et al., 2018) (Vissing et al., 2019). One study has shown that DCM patients with a *TTN*tv are

approximately two to four times more likely to suffer arrhythmias than non-carrier DCM patients (Vissing et al., 2021). In the same study, approximately 1 in 4 patients with TTNtv DCM developed cardiac arrhythmia either before or at the diagnosis of DCM. The position of the TTNtv is important in determining the severity of the arrhythmogenic events. Truncations in exons incorporated into both major cardiac isoforms of titin (N2B and N2BA) and those proximal to the C-terminus cause a greater risk of life-threatening ventricular arrhythmias (Roberts et al., 2015). However, in seeming contradiction to this, TTNtv DCM patients have been shown to present a milder and more treatable form of the disease (Jansweijer et al., 2017). TTNtv DCM patients are at significant risk of developing atrial fibrillation (Ahlberg et al., 2018).

1.8.3.2 Atrial Fibrillation

Atrial fibrillation (AF) is the most common cardiac arrhythmia, affecting approximately 2-4% of the global adult population (Hindricks et al., 2021). AF increases the risk of stroke, heart failure, dementia, depression, hospitalisation and sudden cardiac death (Wolf et al., 1991) (Maisel and Stevenson, 2003) (Waldmann et al., 2020). It is characterised by incidents of disordered and erratic signal propagation in the upper chambers of the heart. AF is classified by the frequency by which it occurs, and usually progresses from paroxysmal (sinus rhythm returns within 7 days without treatment) to persistent (AF lasts longer than 7 days) and permanent AF (AF lasts more than 12 months) (Markides and Schilling, 2003) (Kirchhof, 2017). This phenomenon of AF becoming progressively more frequent is also known as “AF begets AF” and is due to the disease itself promoting further electrophysiological (e.g. shortening of APD and effective refractory period) and structural (e.g. fibrosis and dilatation) changes within the atria that act to stabilise the disease (Wijffels et al., 1995).

There are a variety of acquired and inherited risk factors that predispose individuals to AF. Advancing age as well as environmental triggers such as obesity and alcoholism predispose to AF development. Comorbidities include hyperthyroidism, valve disease (particularly mitral valve disease), heart failure, diabetes, male sex, genetic modifications, hypertension, obstructive sleep apnoea, chronic kidney disease and smoking (Vizzardi et al., 2014) (Lau et al., 2017) (Fabritz et al., 2016). Many of these conditions can promote structural and electrophysiological changes to the atria that ultimately lead to the development and maintenance of AF. For example, obesity can cause chronic inflammation of the heart, which can promote fibrosis in the upper chambers and disrupt signal propagation (Nalliah et al., 2016). Hyperthyroidism is associated with a tachy-cardiomyopathy with shortening of the APD and effective refractory period of atrial cardiomyocytes providing a substrate for the maintenance of AF (Bielecka-Dabrowa et al., 2009). The prevalence of AF will increase approximately two-fold within the next 40 years due to an aging and more comorbid population (Krijthe et al., 2013). AF is a complex cardiac disease that can be caused by a variety of cellular, structural, and electrophysiological changes in the heart. Significant diversity in the aetiology of AF has hampered efforts to define and treat its pathogenesis.

1.8.3.2.1 Pathogenesis of Atrial Fibrillation

AF is initiated and maintained by a ‘trigger’ and ‘substrate’. The trigger is often single or multiple localised groups of cardiomyocytes which develop the ability to generate spontaneous action potentials at a high frequency, collectively known as ectopic foci. The muscular sleeves of the pulmonary veins are a prominent source of ectopic activity, due to key differences in regional electrophysiology and fibre geometry (Khan, 2004). Other sources of triggers away from the pulmonary veins include the superior vena cava, the left atrial appendage, the coronary sinus, the left atrial posterior wall and the ganglionated plexi (Stavrakis and Po, 2017). The substrate can be an electrophysiological and/or structural

characteristics of the atria that maintains AF. The electrical and structural remodelling of the atria into a pro-arrhythmic substrate often occurs concomitantly. Structural remodelling refers to changes in chamber morphology (atrial dilatation) and structure (fibrosis) that disrupt signal propagation (Corradi et al., 2008). Electrical remodelling refers to changes in the intracellular electrophysiology of cardiomyocytes that perturbs normal cardiac activation and conduction. Such changes are usually caused by modifications in ion channel expression and function (Schoonderwoerd et al., 2005).

Maintenance of AF is thought to be caused by the formation of multiple re-entrant circuits producing propagating wavelets at higher rates than the sinoatrial node. Re-entrant circuits, or re-entry, is when propagating electrical impulses circulate within heart tissue (Comtois et al., 2005). Re-entry can occur around a physical barrier such as the vena cava or tricuspid valve (anatomical re-entry) (Frame et al., 1987). It is reliant on the presence of two pathways with differences in conduction velocity or refractory period, as well as unidirectional block. The pathogenesis of AF has long been entrenched in the 'leading-circuit model' which describes how a unidirectional wavefront can cause constant centripetal activation, and therefore refractoriness, of a circuit centre (Waks and Josephson, 2014). This creates a functional barrier in contrast to an anatomical barrier and allows re-entry to sustain itself. The multiple wavelet hypothesis describes how during AF, multiple re-entrant circuits within the atria can work together to continuously re-excite the upper chambers of the heart (Moe and Abildskov, 1959).

Recent computational modelling has shown that particular types of functional re-entry are integral to the perpetuation of AF. Rotors are a type of functional re-entry whereby the wavefront is curved rather than circular (Panfilov and Pertsov, 1982). The wavefront meets the wavetail at a point known as the phase singularity. The velocity of the wavefront is dependent on its curvature, with a large degree of curvature occurring near the phase

singularity. This large curvature causes a slow conduction velocity and makes the area surrounding the phase singularity effectively unexcitable. The phase singularity marks a point around which the wavefront rotates to sustain the rotor. Unlike the leading circle model, the core of the rotor is not refractory and is technically capable of being activated. This difference means that rotors can move through the atria in complex patterns which affect the sustainability of the rotor (Pandit and Jalife, 2013).

AF can be caused by four principal pathomechanisms: structural remodelling, electrical remodelling, intracellular Ca^{2+} handling abnormalities and autonomic nervous system remodelling (Nattel and Harada, 2014). Intracellular Ca^{2+} handling abnormalities pertain to dysfunction in calcium-induced calcium release, which can promote spontaneous SR Ca^{2+} release and delayed afterdepolarisations (DADs) leading to an increased risk of triggered activity (ectopic foci) (Denham et al., 2018). Autonomic nervous system remodelling relates to pathological changes in the sympathetic or parasympathetic regulation of cardiomyocyte stimulation (Linz et al., 2019). It can predispose to shortening of the APD and ERP (predominantly via increased parasympathetic activation), increase the spatial APD/ERP heterogeneity and increase the risk of Ca^{2+} mediated ectopy (Saleeb-Mousa et al., 2023). Key atrial electrical remodelling involves shortening of the atrial APD and hyperpolarisation of the atrial RMP both of which act to shorten atrial refractoriness by increasing the rate of sodium channel recovery and availability. Rises in I_{K1} , I_{KACh} , I_{Kur} , I_{Ks} and reductions in I_{CaL} are all suggested to contribute to this remodelled AF phenotype in humans. In addition, decreases in gap junction expression/activity and/or fibrosis are known to induce significant conduction slowing, increasing the risk of re-entrant circuit formation (Nattel et al., 2020).

1.8.3.2.2 *Risks Associated with Atrial Fibrillation*

AF is a risk factor for various complications including stroke, sudden cardiac death and heart failure (Wolf et al., 1991) (Maisel and Stevenson, 2003) (Waldmann et al., 2020) (Bizhanov

et al., 2023). AF causes a reduction in cardiac output as it promotes rapid and irregular ventricular beating frequencies, valvular regurgitation and disrupted atrial systole (Anter et al., 2009). This reduction in cardiac output can lead to the development of tachycardia related cardiomyopathy and heart failure. Although AF does not directly cause sudden cardiac death, it can trigger life-threatening ventricular arrhythmias and facilitate the development of a substrate susceptible to SCD (Waldmann et al., 2020).

Patients with AF are approximately five-fold more likely to suffer a stroke (Lane et al., 2017). AF is the leading cause of cardioembolic stroke. (Arboix and Alioc, 2010). The mechanisms which underpin how AF causes thrombogenesis are complex and incompletely understood. However, it is known that abnormalities in blood flow, the pooling of blood, alterations in platelet activity and changes to wall structure in the left atrium and appendage can contribute towards thrombus formation (Virchow, 1867) (Kamel et al., 2016). Thrombus formation in the left atrial appendage is largely composed of platelets and fibrin. These may embolize to the cerebral vasculature, leading to occlusion and devastating stroke. Novel oral anticoagulants are primarily used to mitigate the risk of stroke in patients with AF with patients appropriately risk-stratified prior to anticoagulation, with the benefits of stroke prevention and risk of bleeding explained. Risk calculators are used to facilitate this.

1.8.3.2.3 Treatment of Atrial Fibrillation

Treatment for AF is dependent on the frequency of arrhythmia, duration and aetiology and can be broadly categorised according to stroke prevention, rhythm control (maintenance of sinus rhythm) and rate control (regulating heart rate) (Prystowsky et al., 2015) (Wiggins et al., 2024). Stroke prevention, as previously mentioned, can be addressed through prescription of an oral anticoagulant. Risk of bleeding is assessed prior to prescription through a scoring system known as the CHA₂DS₂-VASc score, which takes into account factors including age, sex, diabetes and hypertension (Lip and Lane, 2015). Rate and rhythm control can be treated

with pharmacological or non-pharmacological therapies. Non-pharmacological therapies for rate control include atrioventricular junctional ablation and the implantation of a pacemaker. Catheter ablation and direct current cardioversion are the predominant non-pharmacological treatments for rhythm control. Direct current cardioversion is performed on patients with recurrent and symptomatic AF and involves eliciting a synchronized electrical shock to reset the rhythm of the heart. Catheter ablation is performed at regions which are prominent sources of ectopic activity including the pulmonary veins, superior vena cava, left atrial appendage and ganglionated plexi (Al-Khatib et al., 2014). Pharmacological treatments for rate control can be broadly classified into β -adrenoceptor blockers (class II), calcium channel antagonists (class IV) or other agents (class V). β -adrenoceptor blockers such as Atenolol and Nadolol, which are class II antiarrhythmics, inhibit the sympathetic stimulation of the heart. Calcium channel antagonists, such as Verapamil and Diltiazem, which are class IV antiarrhythmics, impede voltage gated calcium channels to slow conduction through the atrioventricular node (Prystowsky et al., 2015). Class V antiarrhythmics such as Digoxin and Adenosine work to regulate the rate of the heart by a variety of mechanisms.

Pharmacological treatment for rhythm control is dependent on the absence or presence of heart disease (coronary artery disease or heart failure), but broadly involves the administration of class I (sodium channel blockers) or class III (potassium channel blockers) antiarrhythmics. Sodium channel blockers such as flecainide and propafenone, prolong atrial refractoriness by reducing steady state sodium channel availability. Potassium channel blockers such as sotalol, amiodarone and dronedarone, target and prolong phase III repolarisation which again acts to increase refractoriness (An et al., 2018) (Barton et al., 2020). Many of the treatment approaches listed above focus on terminating the arrhythmia or managing the symptoms of the disease. The pharmacological agents prescribed are non-specific in their effect and are broadly palliative rather than preventative. They may cause

deleterious side effects including hypotension, pulmonary fibrosis, AV block and bradycardia (Barton et al., 2020).

Drug trials into novel anti-arrhythmics have failed to provide a new therapeutic in 30 years (Ang et al., 2020). This is partly due to the fact that drivers of AF can substantially differ between patients and are heavily influenced by genetic risk factors and/or co-morbidities. No significant cardiac pathology is observed in 30 % of patients with AF, indicating a strong heritable component to the disease (Chugh et al., 2010). Indeed, early onset AF, which is defined as the diagnosis of AF before the age of 66, has been showed to demonstrate markers of heritability (Ellinor et al., 2010) (Yoneda et al., 2021). Interestingly, numerous GWAS have reported that SNPs adjacent to the *PITX2* gene associate at least 10 to 100-fold more strongly with AF than any other genetic modification (Roselli et al., 2020) (Gudbjartsson et al., 2007). Research into the genes/mechanisms associated with heritable forms of AF may provide novel therapeutics capable of preventing the initial development of the disease in patients with pathogenic variants. Indeed, pharmacological studies have shown that a reduction in *PITX2* expression increases the sensitivity to flecainide and dronedarone, potentially offering a first step towards more personalised AF therapy (Syeda et al., 2016) (Holmes et al., 2021). Numerous other gene variants have been implicated in conferring increased AF risk, including those associated with developmental genes (such as *TBX5* and *ZFHX3*), genes related to cell architecture and communication (such as *GJA1/5* and *NUP155*) and those linked to ion channels (such as *KCNN3*, *KCNA5* and *SCN1-4B*) (Christophersen and Ellinor, 2016). Recent studies have now identified *TTNtv* as a novel genetic risk factor, independent of DCM diagnosis, for the development of early onset AF (Ahlberg et al., 2018). Deciphering the mechanism(s) by which *TTNtv* predisposes to AF development is a key aim of the current thesis.

1.8.3.3 Titin Truncating Variant Associated Atrial Fibrillation

TTNtv are, independent of DCM diagnostic status, enriched in patients diagnosed with both typical and early onset AF (Choi et al., 2018) (Haggerty et al., 2019). Recent genome wide association studies have also formed a robust link between common titin variants and an increased risk of AF (Christophersen et al., 2017) (Nielsen et al., 2018). Variants of titin are enriched in patient cohorts with very early onset AF (< 45 years old) (Goodyer et al., 2019). Furthermore, TTNtv patients with early AF have a 30 % increased risk of developing DCM over those without a variant, indicating that the arrhythmia may play a part in DCM pathogenesis (Barrett et al., 2023). Although DCM patients with a TTNtv have an increased risk of ventricular tachycardia and fibrillation there is no evidence to suggest that TTNtv predispose individuals to ventricular arrhythmia prior to or independent of the onset of DCM (Corden et al., 2019). The novelty of the link between TTNtv and AF means that the mechanism behind it is, as yet abstruse. There is however significant evidence to suggest that structural remodelling of the atria may be important in the propitiation of AF in TTNtv carriers.

1.8.3.4 TTNtv Associated Structural Remodelling of the Atria

Structural remodelling of the atria is characterised by chamber dilatation and fibrosis (Nattel and Harada, 2014). Atrial fibrosis refers to the accumulation of extracellular matrix in the upper chambers of the heart. It promotes AF by disrupting signal propagation through the atria. This leads to conduction heterogeneities and re-entrant circuits. In addition to this, perturbations of cardiomyocyte-cardiac fibroblast coupling caused by fibrosis, promote changes in the intracellular electrophysiology of cardiomyocytes that are pro-arrhythmic (Yue et al., 2011). Evidence of TTNtv mediated remodelling of the atria has been observed in the clinic, as well as in a range of genetically modified animal models. Early onset AF patients carrying TTNtv show an increase in atrial fibrosis (Andreasen et al., 2020).

Furthermore, TTNtv DCM patients demonstrate greater left atrial dysfunction than those without a variant (Henkens et al., 2023). Ahlberg et al., (2018) used a CRISPR-Cas9 engineered zebrafish model to assess the effects TTNtv have on heart development *in vivo*. The zebrafish model demonstrated impaired sarcomere formation, atrial fibrosis, and prolonged PR intervals. A similar zebrafish model, described in Huttner et al., (2018), showed atrial dilation and impaired diastolic function. An AF predisposing TTNtv modelled in mouse demonstrated broadly normal cardiac function as measured by echocardiography, but unorganised sarcomeres and focal fibrosis in the atria (Kalstoe et al., 2023).

The RNA binding protein, RBM20, is a key modulator of *TTN* alternative splicing. Rats with a *RBM20* loss of function *TTN* variant exhibit altered titin isoform expression, fibrosis, widening of the QRS complex and a predisposition to arrhythmia and sudden cardiac death (Guo et al., 2012). Similarly, mice with missense mutations in *RBM20* show significant cardiac fibrosis, upregulation of the pro-fibrotic genes *COL1A2*, *COL3A1*, and *MMP2* and AF (Ihara et al., 2020). *In vitro* studies investigating the role TTNtv have in stem cell derived cardiomyocytes have demonstrated an upregulation in genes associated with remodelling and cardiac fibrosis including *CTGF*, *NPPA* and *NPPB* (Huang et al., 2023). Atrial fibrosis represents a viable mechanism by which TTNtv could cause AF.

1.8.3.5 Hypothesis behind TTNtv Atrial Remodelling

Although titin performs a number of diverse functions within the sarcomeres of cardiac myocytes, its primary roles are to help maintain myofibril passive stiffness and regulate the length of the sarcomere. Previous studies have demonstrated that truncating variants of titin cause the formation of shortened and disorganised sarcomeres (Hinson et al., 2015) (Zaunbrecher et al., 2019) (Ahlberg et al., 2018) (Schick et al., 2018) (McAfee et al., 2021). Furthermore, TTNtvs have been shown to cause a decrease in length-dependent activation of sarcomeres and increased elasticity of the myofibrils (Vikhorev et al., 2020). More elastic

myofibrils, constructed with shortened sarcomeres, likely causes contractile dysfunction and mechanical stress.

Cardiomyocytes under mechanical stress release pro-fibrotic signalling proteins which promote cardiac remodelling through paracrine cardiac fibroblast activation (Heras-Bautista et al., 2019). It is possible that cardiomyocytes carrying a TTNtv undergo increased mechanical stretch and release more pro-fibrotic signals. Such signals may cause the activation of nearby cardiac fibroblasts, promote extracellular matrix deposition, and ultimately lead to the development of fibrosis and an accompanying risk of AF. A diagram illustrating the hypothesis is shown in *Figure 1.7*.

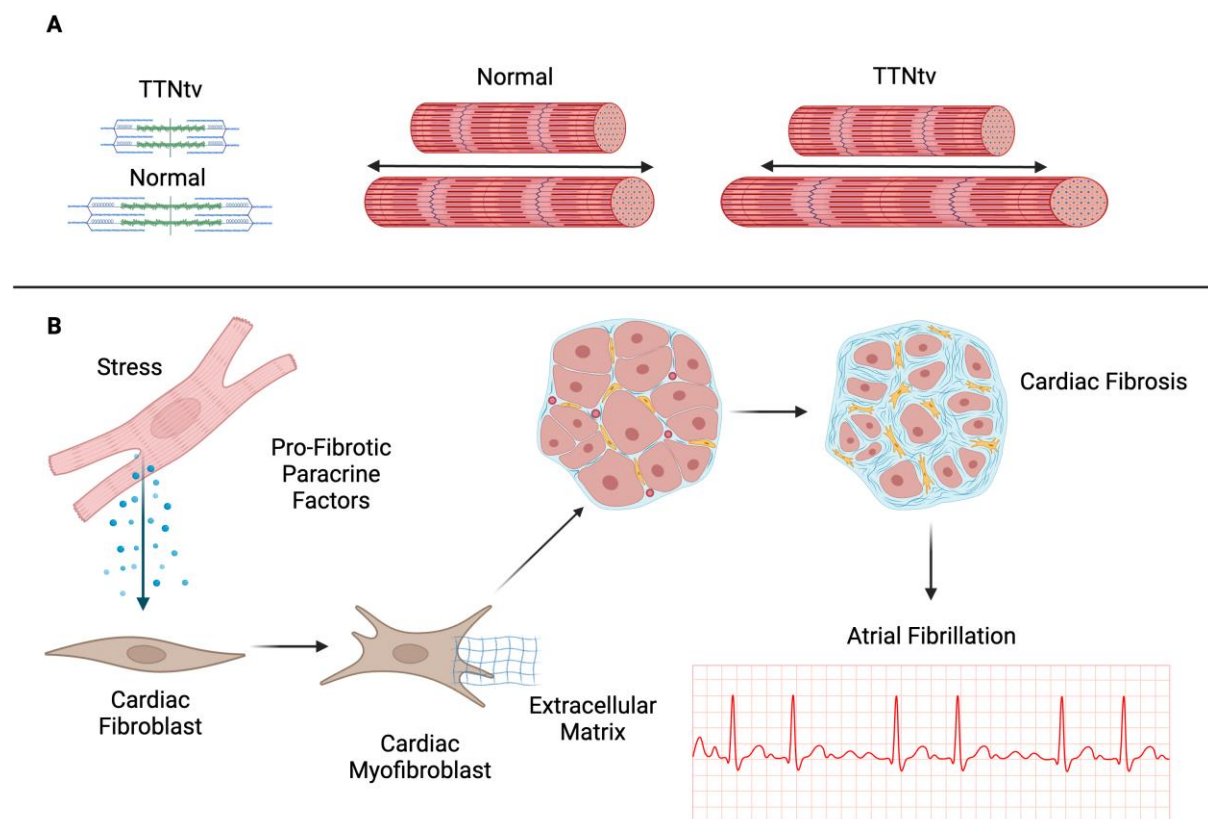


Figure 1.7: TTNtv associated AF Hypothesis

TTNtv have previously been demonstrated to cause shortened sarcomeres and more elastic myofibrils (A). Shortened sarcomeres and increased elasticity in myofibrils may cause increased stretch or stress of cardiomyocytes upon contraction. Increased stress may cause the release of pro-fibrotic signalling proteins such as TGF- β 1. Paracrine signalling proteins may activate nearby cardiac fibroblasts into myofibroblasts and stimulate the release of extracellular matrix. An increase in extracellular matrix protein may cause atrial remodelling and cardiac fibrosis, which may act as a substrate for re-entry and AF (B).

1.8.3.6 Investigating the effect TTNtv have on Atrial Disease

Investigating the ways in which TTNtv increase the risk of AF may improve the accuracy of clinical prognoses. Moreover, a better understanding of the mechanism behind TTNtv associated AF may lead to the refinement of existing clinical care and the development of novel therapeutics. Further work is required to help elucidate the still contested pathomechanism behind TTNtv associated DCM. With DCM characteristically manifesting in the left ventricle, previous studies have focused on the effect TTNtv have in the lower chambers of the heart. However, in many cases of TTNtv DCM pathological effect manifests in the atria prior to the ventricles. This is evidenced by the increased propensity carriers have for AF prior to development of the DCM pathoanatomy (Vissing et al., 2021). This has led to the coining of the term ‘atrial cardiomyopathy’ by some, to describe the early pathological effects of TTNtv (Andersen and Olesen, 2019). Exploring the effect TTNtv have in an *in vitro* atrial model will help elucidate the molecular mechanisms behind TTNtv associated AF. Furthermore, it may provide fresh insight into TTNtv associated DCM.

From an ethical standpoint, animal models, especially mice, are currently bearing a large proportion of the experimental burden in heart disease research despite lacking physiological relevance. Primary cardiomyocytes, derived from patients, are difficult to obtain and can be broadly impractical. Induced pluripotent stem cells (iPSCs) may act as a viable source of atrial cardiomyocytes in which the pathology of TTNtv can be studied.

1.9 Induced Pluripotent Stem Cells

1.9.1 Generation of Cardiomyocytes from Induced Pluripotent Stem Cells

Following the pioneering work by Takahashi and Yamanaka, (2006) in identifying transcription factors capable of inducing pluripotency in somatic cells, cellular reprogramming technology has revolutionised disease modelling. The generation of iPSC lines from genetically diverse individuals has enabled researchers to explore the impact

common and rare genetic variants have on complex disease. The relative ease in which genetic engineering can be performed on iPSCs is unparalleled and facilitates the “high throughput” screening of pathogenic variants such as TTNtv. Cultures of iPSCs can be differentiated into cardiomyocytes by the manipulation of the Wnt signalling pathway. This allows the generation of a variety of cardiac cell types, including ventricular, atrial, and nodal cardiomyocytes (BurrIDGE et al., 2014) (Cyganek et al., 2018) (Schweizer et al., 2017). In contrast to primary cardiac cells, iPSCs act as both a renewable and practical source of cells. Free from the ethical restrictions concomitant with embryonic stem cells and capable of being derived from individuals that vary in age, sex, race, and disease state, the versatility afforded to iPSCs has led to their routine use in cardiac disease modelling and arrhythmia research.

1.9.2 Heart Disease Research using iPSC-derived Cardiomyocytes

The adoption of iPSC models into cardiac disease modelling has not come without challenges. Inefficient differentiation protocols yielding heterogeneous and often phenotypically immature cardiac cells has hindered the utility of iPSCs in modelling many complex cardiovascular diseases (Goedel et al., 2017). Despite this, iPSC-derived cardiomyocytes (iPSC-CMs) have been successfully used to study channelopathies including long-QT and Brugada syndrome (Savla et al., 2014). The monogenic aetiology of many channelopathies means that phenotypic variation can often be adequately assessed in single-cell assays. This circumvents the need for vast quantities of homogenous cardiac myocytes. In contrast to some of the other physiological properties of iPSC-CMs, the activity of many of the key ion currents (inward sodium current, inward calcium current, delayed rectifier current, transient outward current) is broadly similar to human adult cardiomyocytes (Knollmann, 2013). However, changes in the expression of two currents cause severe limitations on how analogous the electrophysiology of iPSC-CMs is to primary cardiomyocytes. Reduced I_{K1} expression causes a depolarised membrane potential, reduced

sodium channel activity and a slow upstroke velocity (Goversen et al., 2018). Whilst the presence of the funny current in iPSC-CMs means that they demonstrate automaticity. Despite this, pathogenic variants identified in patients with cardiomyopathies and/or arrhythmias have been successfully modelled in iPSC-CMs to investigate the molecular mechanisms behind their pathogenesis (Ma et al., 2018).

1.9.3 Challenges with the Utility of iPSC-CMs

The greatest challenge associated with the widespread employment of iPSC models in cardiac disease modelling remains the phenotypic immaturity of the cardiomyocytes derived. This is evidenced by the aforementioned automaticity, low I_{K1} current density and depolarised membrane potential present in many populations of iPSC-CMs (Goversen et al., 2018). The problem is further exacerbated when considering the age-related dependency of many cardiovascular diseases. There is a myriad of methods used to enhance maturation of iPSC-CM. These can range from mechanical and electrical stimulation of the cells to the construction of 3D organoids. Many of these methods are comprehensively described in Machiraju and Greenway (2019).

Current differentiation protocols can generate cells that demonstrate tissue specific expression of atrial, ventricular and nodal ion channels, transporters and connexins (Cyganek et al., 2018) (Schweizer et al., 2017). Current protocols, however, often generate mixed populations of cells. The optimisation of cellular differentiation protocols is often limited by the onerous and expensive nature of cellular differentiation and characterisation. The recent incorporation of genetically encoded calcium sensors into commonly used iPSC lines has helped ameliorate this by facilitating high-throughput phenotypic screening of iPSC-CMs following cellular differentiation (Chen et al., 2017).

Re-entrant arrhythmias, such as AF, often present due to structural differences in the 3D anatomy of the heart. This is challenging to model *in vitro* in 2D monolayers of iPSC-CMs.

The integration of iPSC derived cardiac cells in co-culture and three-dimensional culture systems can provide models that demonstrate significantly greater phenotypic maturity and physiological relevance (Lemoine et al., 2017). However, they are still some way off recapitulating the intricacies of the cardiac micro-anatomy and intra-chamber regional variability which are important to both arrhythmia and heart failure (Holmes et al., 2016). Furthermore, pathophysiological stressors including diabetes, hypertension, hypoxia, ageing, obesity and reduced cardiac blood flow, which act as major drivers for arrhythmogenesis, are difficult to recapitulate, even in 3D iPSC-CM cultures (Chow et al., 2014) (Yildirim et al., 2002) (Morand et al., 2018) (Lau et al., 2013) (Pathak et al., 2015).

1.9.4 Emerging Strategies to Improve iPSC-CM Models

In recent years, an amalgamation between iPSC-based disease modelling and tissue engineering has fathered the generation of three-dimensional iPSC-CM models, such as cardiac microspheres and engineered heart tissues (Beauchamp et al., 2015) (Schaaf et al., 2011). 3D cardiac models have demonstrated improved maturation characteristics including enhanced intracellular calcium handling and increased I_{K1} current densities (Amano et al., 2016) (Silbernagel et al., 2020) (Buikema et al., 2013). A comprehensive description of three-dimensional *in vitro* cardiac models can be obtained from Salem et al., (2021).

The incorporation of co-culture and three-dimensional culture systems into microfluidic “heart on chip” platforms is an exciting prospect. In-built optical and electrical sensors allow data to be generated on calcium handling and contractility (Cho et al., 2020). Furthermore, microfluidic chips enable greater control over culture conditions such as pH and substrate stiffness, with future iterations possibly permitting researchers to adjust parameters to consider pathophysiological stressors including hypoxia and reduced blood flow (Beauchamp et al., 2020).

The maintenance cost required for the use of iPSCs in heart disease and arrhythmia research

is substantially lower than that of maintaining *in vivo* models such as mice and zebrafish. Pathological variants of genes that cause embryonic lethality in mouse models can be modelled in iPSC models without the design and generation of complex conditional expression systems (Nishii et al., 2014). Despite this, there is scepticism about the *in vivo* reproducibility of experimental data derived from iPSC models. Presently, validation of such experimental data is often required in small rodent animals. The development of more efficient differentiation protocols and maturation strategies will likely facilitate the generation of iPSC-CMs that are phenotypically much closer to adult cardiac myocytes. Furthermore, future iterations of co-culture model systems will provide greater accuracy in replicating the heterocellular cell to cell and cell to matrix interactions present in the myocardium during AF and DCM.

1.10 Development of *In Vitro* Modelling Systems

The further development of alternative cellular modelling systems, such as iPSC-CM models, may help reduce *in-vivo* heart disease research. The predominant limitation with the use of stem cell derived cardiac models in disease research is that the cells generated struggle to recapitulate the physiology and microenvironment of primary cardiac tissue (van Mil et al., 2018). Cardiac disease is complex and often manifests due to pathological changes in multiple cell types. The electrical and mechanical interplay present between cardiomyocytes and the stromal cells, cardiac fibroblasts, is abstruse but integral to the functioning of the myocardium in health and disease (Pellman et al., 2016). Induced pluripotent stem cell derived cardiac fibroblasts (iPSC-CFs) have emerged as a novel *in vitro* model that can be used to recapitulate and study aspects of cardiac fibrosis (Zhang et al., 2020). However more work is required to understand how analogous the cells are to primary cardiac fibroblasts. The generation of 3D cardiac models has enabled researchers to investigate complex cardiac diseases which involve cell-cell and cell to matrix interactions. The incorporation of non-

myocyte cell types, such as iPSC derived cardiac fibroblasts, into 3D cardiac models will further increase our capacity to study complex cardiac diseases such as AF and cardiomyopathy (Windt et al., 2023).

1.11 Hypotheses and Aims

1.11.1 The Generation and Validation of Atrial iPSC-CMs

Hypothesis: Atrial iPSC-CMs can be used as an experimental system to study atrial disease.

Aims:

1. Optimise the differentiation of atrial cardiomyocytes from iPSCs by determining an effective concentration of retinoic acid to add (*Chapter 2*)
2. Characterise the atrial iPSC-CMs for expression of compartment specific markers by qPCR, western blotting, immunofluorescence and patch clamp analysis (*Chapter 2*)

1.11.2 The Generation of a 3D Co-Culture iPSC-CM/iPSC-CF Model

Hypothesis: The addition of Induced Pluripotent Stem Cell Derived Cardiac Fibroblasts into a 3D cardiac model will improve its physiological relevance and utility

Aims:

1. Generate engineered heart tissues with and without the addition of iPSC-CFs (*Chapter 3*)
2. Demonstrate that the addition of iPSC-CFs into the EHTs is beneficial to the contractile function of the model (*Chapter 3*)

1.11.3 TTNtv and Electrical Remodelling

Hypothesis: TTNtv causes changes in the intracellular electrophysiology of atrial iPSC-CMs that cause AF

Aims:

1. Characterise spontaneous and stimulated action potentials derived from atrial iPSC-CMs with a TTNtv using current clamp (*Chapter 4*)
2. Investigate whether there are any changes in inward sodium or outward potassium current in atrial TTNtv iPSC-CMs using voltage clamp (*Chapter 4*)
3. Explore any electrophysiological changes observed with follow up experiments and/or *in silico* modelling (*Chapter 4*)

1.11.4 TTNtv and Structural Remodelling

Hypothesis: TTNtv causes structural remodelling of the atria, which promotes AF

Aim:

1. Assess whether TTNtv cause the formation of shortened sarcomeres in atrial iPSC-CMs using immunofluorescence (*Chapter 4*)
2. Assess whether TTNtv atrial iPSC-CMs demonstrate perturbed contractile function (*Chapter 4*)
3. Investigate whether cardiac stress markers are upregulated in TTNtv atrial iPSC-CMs using qPCR and ELISAs (*Chapter 4*)
4. Ascertain the effect a TTNtv has on extracellular matrix gene and protein expression in a co-culture EHT model (*Chapter 4*)

1.11.5 Cardiac Fibroblasts

Hypothesis: iPSC-CFs will be analogous to primary cardiac fibroblasts and act as a suitable model to investigate the electrophysiological changes that occur in cardiac fibroblasts during activation

Aim:

1. Generate iPSC-CFs and compare their plasticity for activation against primary ventricular and atrial cardiac fibroblasts using immunofluorescence and qPCR (*Chapter 5*)
2. Explore their utility as a model of cardiac fibrosis by investigating changes in ion channel subunit gene expression using qPCR (*Chapter 5*)
3. Perform RNA-seq and quantitative proteomics to identify changes in ion channel expression pivotal to cardiac fibroblast activation (*Chapter 5*)
4. Validate changes seen in ion channel expression using voltage clamp

Chapter 2: Generation and Validation of a Human Atrial iPSC- CM Model

Parts of the materials and methods section of this chapter were taken from previously published work: Cumberland, M.J., Euchner, J., Azad, A.J., TN Vo, N., Kirchhof, P., Holmes, A.P., Denning, C. and Gehmlich, K., 2023. Generation of a human iPSC-derived cardiomyocyte/fibroblast engineered heart tissue model. *F1000Research*, 12, p.1224.

2.1 Introduction

2.1.1 TTNtv and Atrial Disease

Understanding the ways in which TTNtv increase the risk of AF may improve the accuracy of prognoses for patients, lead to the refinement of existing care and further the development of novel therapeutics. Moreover, it may help elucidate the complex pathomechanism(s) behind TTNtv associated DCM. With DCM manifesting characteristically in the left ventricle, previous studies using iPSC-CMs have predominantly focused on exploring the mechanical disruption caused by TTNtv in the sarcomeres of ventricular iPSC-CMs. Patients with TTNtv associated DCM often first clinically present with pathological defects, namely arrhythmia, in the atria rather than the ventricles (Vissing et al. 2021). This is in agreement with previous studies on TTNtv in animal models, where atrial dilatation and fibrosis can be some of the first symptoms (Ahlberg et al., 2018) (Huttner et al., 2018). This has led members of the field to consider whether the pathology of TTNtv can be described as an atrial cardiomyopathy (Fatkin et al., 2019) (Vad et al., 2020). Furthermore, and as previously discussed in 1.8.3.3, TTNtv can predispose individuals to AF independently from the development of DCM. The greatest problems with the implementation of stem cell technology in cardiac disease modelling are the immaturity and heterogeneity of the cells derived. This is especially pertinent when considering cardiovascular diseases, which can be age-dependent and compartment specific.

2.1.2 Atrial Cardiomyocyte Differentiation

Early attempts differentiating cardiomyocytes from stem cells yielded heterogenous populations of sinoatrial, ventricular, and atrial like cells (Zhang et al., 2002) (Kolossoff et al., 2005) (Maltsev et al., 1993). Gassanov et al., (2008) and Zhang et al., (2010) showed that manipulation of the retinoid signalling pathway, through the addition of retinoic acid, could drive the differentiation of embryonic stem cells into atrial-like cardiomyocytes with

compartment specific expression of the myosin light chain (MLC-2a) and natriuretic peptide (ANP). Later studies demonstrated that embryonic stem cell derived atrial cardiomyocytes showed expression and function of atrial specific ion channels including $K_v1.5$ (*KCNA5*) and $K_{ir3.1}$ (*KCNJ3*). Expression of these and other ion channels caused atrial-like action potentials with slowed upstroke velocities and shortened durations (Devalla et al., 2015). A comprehensive study phenotyping the transcriptome, proteome, intracellular electrophysiology, calcium handling and contractile function of atrial iPSC-CMs supported their application in compartment specific disease modelling (Cyganek et al., 2018). A robust and relatively mature atrial iPSC-CM model is required to assess the impact pathogenic variants such as *TTNtv* have in human atria. The following chapter entails the generation and validation of such a model.

2.1.3 Hypothesis and Aims

Hypothesis: The addition of retinoic acid during iPSC cardiac differentiation will cause the generation of atrial like cardiomyocytes that demonstrate compartment specific electrophysiological, genetic, and structural markers.

Aim: Generate and validate the use of atrial iPSC-CM

Objectives:

1. Evaluate compartment specific gene expression following the addition of 0.5 and 1.0 μM retinoic acid
2. Evaluate expression of compartment specific structural and ion channel subunit genes in atrial iPSC-CM by qPCR
3. Evaluate protein expression of compartment specific structural markers in atrial iPSC-CMs
4. Evaluate the intracellular electrophysiology of atrial and ventricular iPSC-CMs

2.2 Materials and Methods

2.2.1 Cell Culture

2.2.1.1 Induced Pluripotent Stem Cell Culture

All iPSC culture was performed using the HPSI0114i-kolf_2 line commonly known as KOLFC2 (http://www.iPSCi.org/lines/#/lines/HPSI0114i-kolf_2). iPSCs were cultured in mTeSR Plus media (StemCell Technologies, 100-0276). Media was changed every 48 hours until cells reached 80 % confluency. All iPSCs were maintained in 6 well plates pre-coated with 1:100 Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific, A1413201). Coating of plates was performed according to the manufacturer's protocol. All cells were incubated in a 37 °C and 5 % CO₂ incubator. Cells were dissociated at 80 % confluency by treating with TrypLE™ Express Enzyme (1X), phenol red (Thermo Fisher Scientific, 12605010) for 3 minutes at 37 °C and 5 % CO₂. The TrypLE was removed from the wells of the plate, and the cells were gently washed off with mTeSR Plus media. Cells were counted using a Neubauer and were plated at a given density (normally between 10,000 and 20,000 cells/cm²) in mTeSR Plus medium, supplemented with 10 µM of ROCK inhibitor Y-27632 (Selleck, S1049). Media was changed the day after passaging to ensure removal of Y-27632.

2.2.1.2 iPSC-CM Differentiation

iPSCs were differentiated into cardiomyocytes using a protocol broadly described in Smith et al., (2018) and Cumberland et al., (2023). A schematic of the protocol can be seen in *Figure 2.1*. Undifferentiated iPSCs were seeded onto Corning™ Costar™ 6-well plates (Fisher Scientific, 10578911) coated in geltrex. When cells reached approximately 60 % confluency (*Day -1*) the medium was changed from mTeSR Plus medium to StemPro™-34 SFM (Thermo Fisher Scientific, 10639011) supplemented with Recombinant Human BMP-4 Protein (1 ng/mL) (R&D Systems, 314-BP-010/CF), L-Glutamine 2 mM (Thermo Fisher

Scientific, 25030024) and geltrex (1:100). The media was changed the next day into StemPro™-34 SFM supplemented with L-Glutamine (2 mM final concentration), Recombinant Human BMP-4 Protein (10 ng/mL final concentration) and Human Activin A Recombinant Protein (Thermo Fisher Scientific, PHC9564) (8 ng/mL final concentration) (*Day 0*). After 48 hours, media was changed to RPMI 1640 Medium (Thermo Fisher Scientific, 21875034) with B-27™ Supplement, minus insulin (Thermo Fisher Scientific, A1895601) (1:50), XAV 939 (10 µM final concentration) (TOCRIS, Cat. No. 3748) and KY02111 (10 µM final concentration) (TOCRIS, Cat. No. 4731) (*Day 2*). Media was changed 48 hours after that with RPMI 1640 supplemented with B-27™ Supplement (50X), serum free (Thermo Fisher Scientific, 17504044) (1:50), XAV 939 (10 µM final concentration) and KY02111 (10 µM final concentration) (*Day 4*). Media was changed 48 hours after with RPMI 1640 supplemented with B-27™ Supplement (1:50) (*Day 6*). Media was changed on the cells every 48 hours until the cells were passaged or harvested. Generation of iPSC derived atrial cardiomyocytes was achieved through the addition of retinoic acid (Sigma-Aldrich, R2625) at days 4 and 6 (0.5 or 1.0 µM final concentration). At day 12 after the initiation of differentiation, glucose starvation was performed by changing the media on the cells with RPMI 1640 Medium, no glucose (Thermo Fisher Scientific, 11879020) supplemented with B-27™ Supplement (1:50) for 48 hours.

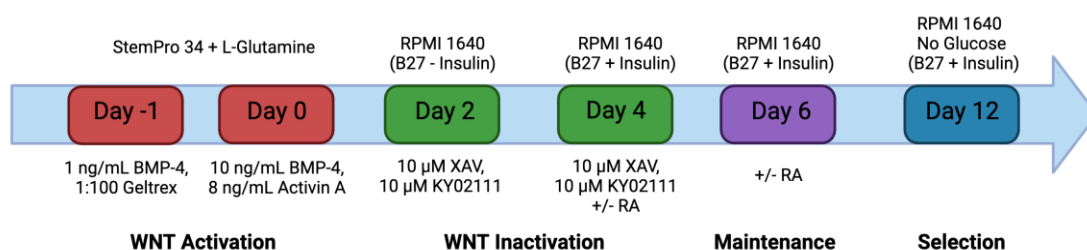


Figure 2.1: Differentiation of iPSC-CMs

Schematic representation of the iPSC-CM differentiation protocol. Mesodermal specification is achieved through the activation of the Wnt signalling pathway using the recombinant proteins: Bone morphogenic protein- 4 (BMP-4) and Activin A. Subsequent inactivation of the Wnt signalling pathway by small molecules XAV 939 and KY02111 enables the

differentiation of cardiac progenitor cells into iPSC-CMs. Differentiation can be driven towards atrial cardiomyocyte specification through the addition of retinoic acid on days 4 and 6. The medium on the cells was changed every 48 hours after day 6 with RPMI 1640 with B27 + Insulin. Selection is achieved from days 12-14 by culturing the cells in RPMI 1640 No Glucose with B27 + insulin for 48 hours.

2.2.1.3 Harvesting of iPSC-CMs for RNA

iPSC-CMs were dissociated and spun down into pellets 30 days after the initiation of differentiation, unless otherwise stated. Cells were washed with PBS and subsequently dissociated by incubating in TrypLE™ Select Enzyme (10X), no phenol red (Thermo Fisher Scientific, A1217701) for 30 minutes at 37 °C. The TrypLE was removed and the cells were washed off with PBS and spun down at 300 g for 3 minutes. The cells were washed again in PBS and spun down at 13,000 g for 3 minutes. The PBS was removed, and the dry pellet was frozen at -80 °C.

2.2.1.4 Passaging of iPSC-CMs for Patch Clamp Analysis and Immunofluorescence

iPSC-CMs were washed with PBS and incubated in TrypLE Select for 30 minutes at 37 °C. The TrypLE was removed and the cells were washed off with pre-warmed DMEM-F12 and spun down at 100 g for 10 minutes. Cells were resuspended in plating media consisting of RPMI 1640, B27 Supplement (1:50), KnockOut™ Serum Replacement (10 %) (Thermo Fisher Scientific, 10828028) and Thiazovivin (8 µM) (StemCell Technologies, 100-0247). iPSC-CMs were seeded onto Corning™ BioCoat™ 12mm #1 German Glass Coverslips (Fisher Scientific, 10468681), which had been individually inserted into wells of a Corning 24 well plate, and previously sterilised and pre-coated in 1:100 Geltrex. 50,000 iPSC-CMs were seeded in each well of the 24 well plate to ensure single cells for patch clamp and immunofluorescence. Media was changed the following day to RPMI 1640 with B27 Supplement (1:50). Passaging occurred 27 days after the initiation of differentiation to give the cells 3 days to recover prior to use in experiments (Day 30).

2.2.2 Immunofluorescence

2.2.2.1 Fixing and Staining

Cells were fixed for 14 minutes at room temperature using 4 % paraformaldehyde solution (PBS) (Thermo Fisher Scientific, J19943.K2). Fixed cells were blocked for 1 hour in blocking buffer consisting of PBS, Fetal Bovine Serum (5 %), Bovine Serum Albumin (1 %) (Merck Millipore, A9418-10G) and Triton™ X-100 (0.5 %) (Merck Millipore, X100-100ML). Primary antibodies were added at dilutions listed in *Table 2.1*, in blocking buffer for 2 hours at room temperature. This was followed by 3 washes in blocking buffer and the addition of the secondary antibodies (*Table 2.1*) for 1 hour in the dark at room temperature. DAPI (Thermo Fisher Scientific, D1306) was added with the secondaries at 0.1 µg/mL to visualise nuclei. Samples were washed 3 times in blocking buffer and then once with PBS. Samples were mounted onto cover slides using Hydromount Histology Mounting Media (Scientific Laboratory Supplies, NAT1324). Samples were subsequently imaged using a Zeiss LSM780 Confocal laser scanning microscope equipped with a C-Apochromat 63x/1.20 W or C-Apochromat 10x/0.45 W objective.

Table 2.1: Immunofluorescence Antibodies

Target	Company, Catalog no	Host	Concentration
MLC-2a (Primary)	Synaptic Systems, 311 011	Mouse	1:500
MLC-2v (Primary)	Proteintech, 10906-1-AP	Rabbit	1:500
Phalloidin (F-Actin) (Conjugated)	Thermo Fisher Scientific, A22284	N/A	1:500
α-actinin (Primary)	Abcam, ab68167	Rabbit	1: 500
Alexa Fluor™ 488 anti-Mouse IgG (Secondary)	Thermo Fisher Scientific, A28175	Goat	1:1000
Alexa Fluor™ 546 anti-Rabbit IgG (Secondary)	Thermo Fisher Scientific, A-11035	Goat	1:1000
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Nuclear Stain) (Conjugated)	Thermo Fisher Scientific, D1306	N/A	0.1 µg/mL

2.2.2.2 *Sarcomere Analysis*

Sarcomere analysis was performed on images acquired from ventricular and atrial iPSC-CMs 30 days after the initiation of differentiation, stained for α -actinin and phalloidin (F-Actin). Images were acquired from 3 differentiation batches of ventricular and atrial iPSC-CMs. Images were converted into tifs and analysed using the automated z-disc mapping software described in Morris et al., 2020. Mean sarcomere length and orientation order parameter were plotted for each cell type.

2.2.3 RT-qPCR

2.2.3.1 *RNA Extraction and Isolation*

Previously harvested dry cell pellets, stored at -80 °C, were thawed on ice and subsequently processed. Homogenisation of samples was performed using QIAshredder (QIAGEN, 79656). RNA isolation was performed using a RNeasy Mini Kit (QIAGEN, 74104) and performed according to the protocol stipulated by the manufacturer. RNA concentration and purity was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, ND-2000).

2.2.3.2 *cDNA Conversion*

Following quantification, 200 ng of RNA was converted into cDNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Fisher Scientific, 10400745) to give cDNA samples at 10 ng/μl. Reaction mixtures and thermal cycling conditions were respectively prepared and set up as outlined within the product information sheet provided.

2.2.3.3 *Quantitative Polymerase Chain Reaction Setup (qPCR)*

Two types of qPCR setups were used during this chapter. TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, 4444556) was used in combination with Taqman probes, with reactions setup in accordance with the included protocol. 10 ng of cDNA was included within each reaction. The TaqMan™ Gene Expression Assays (FAM) (Applied Biosystems,

4331182) are listed in *Table 2.2*. Taqman reactions were multiplexed with Human GAPD (GAPDH) Endogenous Control (VIC™/TAMRA™ probe, primer limited) (Thermo Fisher Scientific, 4310884E) to allow determination of relative gene expression.

The other type of qPCR setup utilised PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25741) in accordance with the included product data sheet. 10 ng of cDNA was included within each reaction. 800 nM of forward and reverse primer were included in each reaction. KiCqStart® SYBR® Green Primers (Sigma Aldrich, KSPQ12012) were used in all reactions and are listed in *Table 2.3*.

Table 2.2: Taqman Probes

Gene Name	Assay ID
ACTN2	Hs00153809_m1
MYH6	Hs01101445_gH
MYH7	Hs01110632_m1
MYL7	Hs01085598_g1
MYL2	Hs00166405_m1
NPPA	Hs00383230_g1
TTN	Hs00399225_m1
GAPDH	Thermo Fisher Scientific, 4310884E

Table 2.3: SYBR Green qPCR Primers

Gene	Sequence (5' – 3')	Tm (°C)
SCN5A	F – TCT TCA CAG GCG AGT GTA TTG	54.7
	R – CAC GAT GAG GAA GGA GAT GAT G	54.9
KCNJ3	F – AAA AAC GAT GAC CCC AAA GA	52.4
	R – TGT CGT CAT CCT AGA AGG CA	55.4
KCNJ5	F – GGA CAC CCC AGA AGT TAG CA	56.8
	R – CCA ATC TCC ATG TCC TGG TT	54.6
KCNA4	F – GTC CGT CCT GGT CAT CTT AAT C	54.9
	R – CAG CGA ACC ACA AAC TCA AAG	54.7
KCNA5	F – CGA GGA TGA GGG CTT CAT TA	54.1
	R – CTG AAC TCA GGC AGG GTC TC	57.3
KCNH2	F – CTT CCT ATG TCT GGT GGA TGT G	54.9
	R – TGTGGG TTC GTC CCT TTA TC	54.8
GAPDH	F – GAT TCC ACC CAT GGC AAA TTC	54.6
	R – GTC ATG AGT CCT TCC ACG ATA C	54.8

2.2.3.4 qPCR Procedure and Analysis

All qPCRs were performed in either a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (Thermo Fisher Scientific, 4346906) or a MicroAmp™ Optical 384-Well

Reaction Plate with Barcode (Thermo Fisher Scientific, 4309849). Plates were briefly spun down, prior to the initiation of the reaction. Reactions were performed in either an Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, 4351106) or QuantStudio™ 7 Flex Real-Time PCR System, 384-well, desktop (Thermo Fisher Scientific, 4485701). Melt Curves were performed at the end of reactions involving the using of PowerUp™ SYBR™ Green Master Mix to check specificity of the amplicon. Analysis of qPCR data was performed using the Livak method outlined in Livak and Schmittgen, (2001). The housekeeping gene *GAPDH* was used to normalise gene expression in all qPCRs. 3 technical replicates were performed for each sample.

2.2.3.5 *GTExPortal*

The data used for the analyses described in this manuscript were obtained from atrial appendage and left ventricle on the GTEx Portal on 07/11/23.

<https://www.gtexportal.org/home/>

2.2.4 Western Blot

2.2.4.1 *Sample Preparation*

Previously harvested iPSC-CM pellets, stored at -80 °C, were thawed on ice. Following thawing, 40 µl of Novex™ Tricine SDS Sample Buffer (1X) (Thermo Fisher Scientific, LC1676) was added, and the samples were incubated at 100 °C for 3 minutes. The samples were sonicated for approximately 10 seconds using a Fisherbrand™ Q500 Sonicator with Probe (Fisher Scientific, 12893543).

2.2.4.2 *Quantification of Protein Concentration*

Protein concentration was estimated by running a Coomassie stained gel. Briefly, samples were run on a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, 15-well, 15 µl (BIO-RAD, 4561086) gel for 30 minutes at 140 Volts. Protein on the gel was stained using Coomassie Brilliant Blue R-250 Dye (Thermo Fisher Scientific, 20278) (1g Coomassie, 65%

H₂O, 30% methanol, 5% Acetic acid) and protein concentration was estimated by pixel density using ImageJ Software.

2.2.4.3 Immunoblotting

Following relative quantification of protein concentration, the protein samples were run on a 4–15% Mini-PROTEAN® TGX™ Precast Protein Gel at 70 Volts for 1 hour. The Precision Plus Protein Dual Color Standards, 500 µl (BIO-RAD, 1610374) protein ladder was run on each gel to enable estimation of protein size. The gel was transferred to a nitrocellulose membrane using an iBlot™ 2 Gel Transfer Device (Thermo Fisher Scientific, IB21001). The membrane was stained with Ponceau S Solution (Abcam, ab270042) according to the manufacturer's protocol booklet, to visualise total protein. Membranes were blocked in 5 % Milk powder in Tris-Buffered Saline (TBS) (Sigma Aldrich, 1706435) (TBS) 5 % TWEEN® 20 (Sigma-Aldrich, P1379-500ML) (TBST) for 1 hour. Primary antibodies were added to 5 % Milk TBST, at dilutions specified in *Table 2.4*, overnight at 4 °C. Secondary antibodies were added to 1% Milk TBST and incubated at room temperature for 1 hour. The secondary antibodies, with the respective dilutions used, are shown in *Table 2.4*. Protein visualisation was performed using an Odyssey Fc Imaging System (LI-COR) at 800 nm.

Table 2.4: Western Blot Antibodies

Target	Host	Company	Dilution
GAPDH	Rabbit	Cell Signalling Technology, D1H11	1:1000
MLC-2v	Rabbit	Proteintech, 10906-1-AP	1:500
Anti-Rabbit IgG	Goat	LI-COR, 926-32211	1:5000

2.2.5 Patch-Clamp Analysis

2.2.5.1 Patch Clamp Setup

Recordings and analysis were performed using an Axopatch 200B amplifier (Molecular Devices, USA). Analog signals were converted at 50 kHz using a CED micro1401 operated using Signal v6 software (Cambridge Electronic Design, Cambridge, UK).

2.2.5.2 Action Potential Measurements in Ventricular and Atrial iPSC-CMs

Stimulated action potentials were recorded from a single differentiation batch of ventricular and atrial iPSC-CMs at 30 days after the initiation of differentiation. Action potentials were recorded at 37 °C using an internal solution of pH 7.2 (adjusted using KOH). The solution contained (in mM): KCl 135, NaCl 10, MgATP 5, HEPES 10, EGTA 0.1. Micropipette resistances were between 3-4 MΩ. Extracellular solution contained (in mM): NaCl 145, KCl 5.4, MgSO₄·7H₂O 0.83, NaH₂PO₄ 0.33, HEPES 5, Glucose 11, CaCl₂ 1.8, pH 7.4 (NaOH). Action potentials were recorded at 37° C. A 60 second period after breakthrough allowed cell stabilisation. A continuous hyperpolarising current was then applied to the cells to hold the diastolic membrane potential at – 75 mV prior to recording of stimulated action potentials (Workman et al., 2001) (Holmes et al., 2021). Only those cells that required a hyperpolarising current of less than 150 pA were used (Workman et al., 2001). Action potentials were stimulated using a 1 nA, 2 ms current injection at a frequency of 1 Hz (Holmes et al., 2021). 10 successive stimulated action potentials were analysed from each cell using custom algorithms developed in MatLab.

2.2.6 Statistical Analysis

All statistics was performed using GraphPad Prism Version 10.0.03. Gaussian distribution was tested using a Shapiro-Wilk normality test. Either an unpaired t-test with a Welch's correction or an ordinary one-way ANOVA was used to test significance.

2.3 Results

2.3.1 Expression of Atrial and Ventricular Markers in Day 20 iPSC-CMs

2.3.1.1 Optimisation of Retinoic Acid Concentration

The generation of atrial iPSC-CMs was achieved through the addition of retinoic acid on days 4 and 6 of differentiation. There was no previously established protocol for atrial iPSC-CM generation in the laboratory group. Therefore, optimisation of the protocol was required.

Retinoic acid was added to give a final concentration of either 0.5 or 1.0 μM (*Figure 2.2*) on days 4 and 6 of cardiac differentiation. A single batch of cardiomyocytes was matured to 20 days after the initiation of cardiac differentiation and harvested for RNA isolation. qPCR was performed using one differentiation batch (three technical replicates) to ascertain the optimum concentration of retinoic acid required to generate atrial iPSC-CMs.

2.3.1.2 Gene Expression of Structural Cardiac Markers of Day 20 iPSC-CMs

The expression of the following cardiac structural genes was assayed in putatively atrial (0.5 or 1.0 μM RA treated) and ventricular iPSC-CMs: myosin heavy chain 7 (*MYH7*), myosin heavy chain 6 (*MYH6*), myosin light chain 7 (*MYL7*), myosin light chain 2 (*MYL2*), actinin alpha 2 (*ACTN2*) and titin (*TTN*). The *MYH7* and *MYL2* genes respectively encode the ventricular isoforms of the myosin heavy and light chains. The *MYH6* and *MYL7* genes encode the atrial isoforms. The expression of structural cardiac genes in atrial and ventricular iPSC-CMs at 20 days after the initiation of cardiac differentiation is shown in *Figure 2.2*.

Significantly greater expression of the ventricular isoform *MYH7* was observed in the cells treated with 0.5 μM retinoic acid. Expression of the atrial isoform *MYH6* was highest in the cells treated with 1.0 μM retinoic acid and lowest in those treated with 0.5 μM retinoic acid. Expression of the ventricular isoform *MYL2* was significantly downregulated in cells treated with 1.0 μM RA. Furthermore, treatment with retinoic acid caused a significant increase in the expression of the atrial isoform *MYL7*. Expression of titin was significantly lower in the

cells treated with 0.5 μ M RA, but comparable in those treated with no RA or 1.0 μ M RA.

The expression of *ACTN2* significantly decreased when the cells were treated with 1.0 μ M retinoic acid.

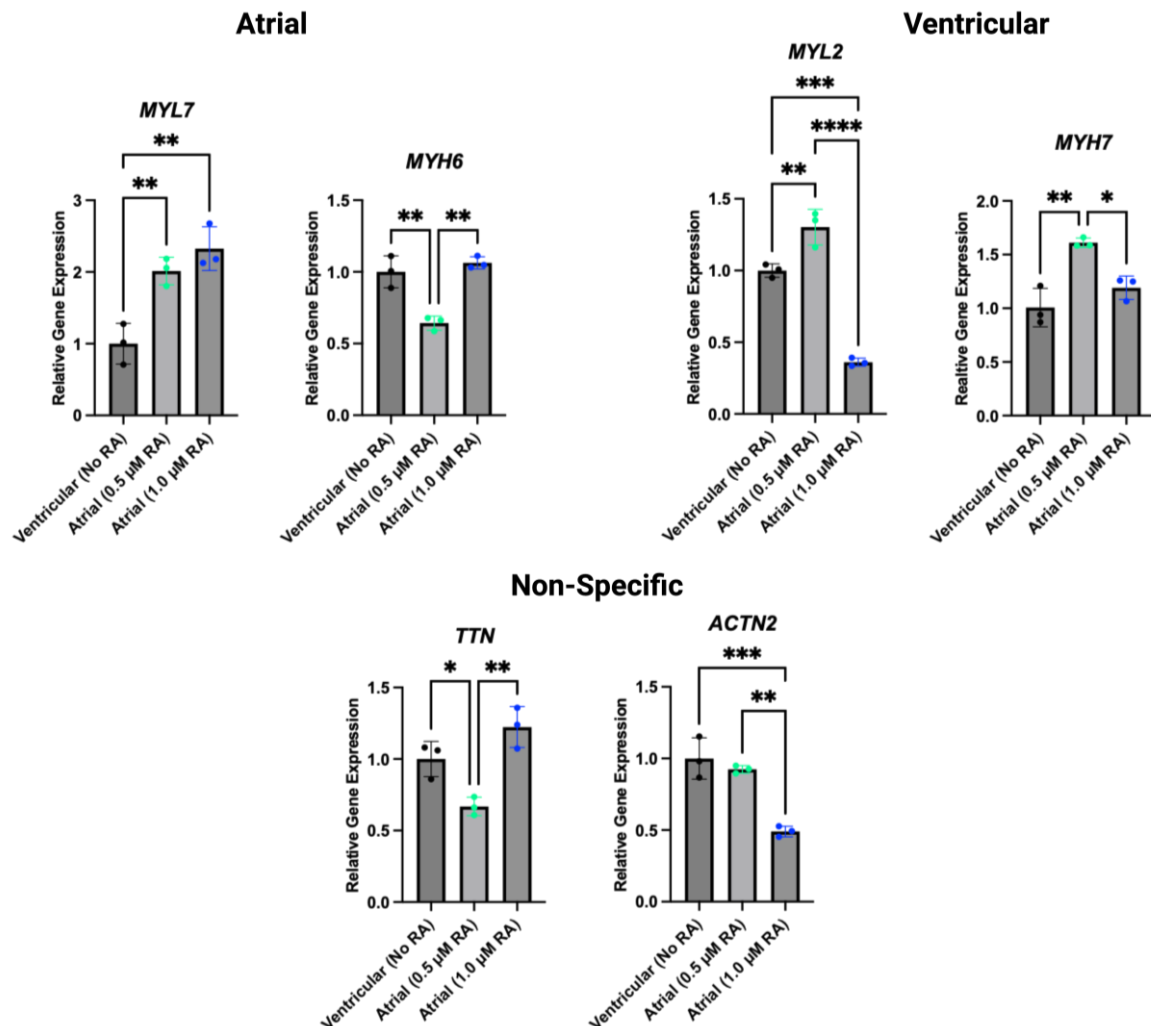


Figure 2.2: Gene Expression of Structural Cardiac Markers in Atrial and Ventricular iPSC-CMs (Day 20)

qPCRs were performed for the cardiac genes myosin heavy chain 7 (*MYH7*), myosin heavy chain 6 (*MYH6*), myosin light chain 7 (*MYL7*), myosin light chain 2 (*MYL2*), actinin alpha 2 (*ACTN2*) and titin (*TTN*). Cells were either treated with no retinoic acid (Ventricular), 0.5 μ M retinoic acid (Atrial) or 1.0 μ M retinoic acid (Atrial). The expression levels of all the genes were normalised to *GAPDH* expression. Mean expression levels and SD are shown in the graph above. An ordinary one-way ANOVA was used to test significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ ($N = 1$, $n = 3$).

2.3.1.3 Gene Expression of Ion Channel Subunit Genes of Day 20 iPSC-CMs

Cells were treated with either 0.5 or 1.0 μ M retinoic acid to induce atrialisation. Expression of the following cardiac ion channel genes was assayed in atrial and ventricular iPSC-CMs:

potassium voltage-gated channel subfamily A member 5 (*KCNA5*), potassium voltage-gated channel subfamily A member 4 (*KCNA4*), potassium inwardly rectifying channel subfamily J member 3 (*KCNJ3*), potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*) and sodium voltage-gated channel alpha subunit 5 (*SCN5A*). To decide which ion channel genes are good markers for atrial or ventricular cardiomyocytes, the relative expression of the ion channel subunit genes listed above was ascertained for human atrial appendage and left ventricle tissue samples (*Table 2.5*). This data was taken from GTExPortal.

Table 2.5: Relative Gene Expression of Ion Channel Subunit Genes

Gene	Gene Expression (Median Tags Per Million)	
	Left Ventricle	Atrial Appendage
<i>KCNA5</i>	1.73	35.57
<i>KCNA4</i>	0.75	0.27
<i>SCN5A</i>	35.11	36.13
<i>KCNJ3</i>	0.15	12.85
<i>KCNJ5</i>	1.80	6.14
<i>KCNH2</i>	26.58	36.92

The expression of ion channel subunit genes in atrial and ventricular iPSC-CMs at day 20 after the initiation of cardiac differentiation is shown in *Figure 2.3*. Expression of the atrial specific ion channel subunit gene *KCNA5* was higher in the ventricular iPSC-CMs than in the ones treated with 0.5 μ M and 1.0 μ M RA retinoic acid. However, a significant increase in expression was observed between the cells treated with 0.5 μ M and 1.0 μ M RA. Treatment with retinoic acid caused a significant decrease in expression of the ion channel subunit gene *KCNA4*. Furthermore, the atrial specific *KCNJ3* gene was strongly upregulated in the cells treated with 1.0 μ M retinoic acid. Low expression of the predominantly atrial specific gene *KCNJ5* was seen in cells treated with 0.5 and 1.0 μ M RA. There was no detectable expression of *SCN5A*. Overall, the 1.0 μ M RA treated iPSC-CMs demonstrated more atrial

like gene expression pattern (upregulation of *MYH6*, *MYL7* and *KCNJ3*) and was therefore used in future experiments.

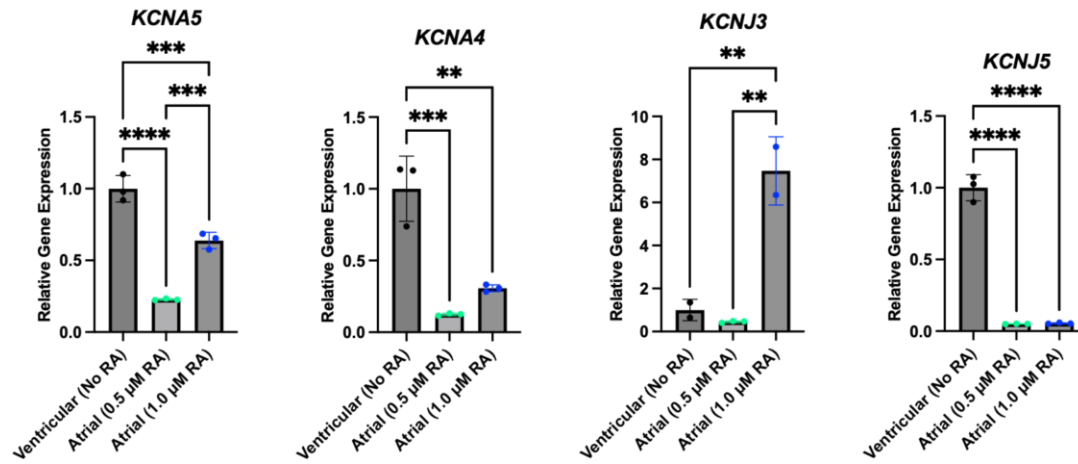


Figure 2.3: Gene Expression of Ion Channel Subunit Genes in Atrial and Ventricular iPSC-CMs (Day 20)

qPCRs were performed for the ion channel subunit genes: potassium voltage-gated channel subfamily A member 5 (*KCNA5*), potassium voltage-gated channel subfamily A member 4 (*KCNA4*), potassium inwardly rectifying channel subfamily J member 3 (*KCNJ3*) and potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*). Cells were either treated with no retinoic acid (Ventricular), 0.5 μ M retinoic acid (Atrial) or 1.0 μ M retinoic acid (Atrial). The expression levels of all the genes were normalised to *GAPDH* expression levels. Mean expression and SD are shown on each graph. An ordinary one-way ANOVA was used to test significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ ($N = 1$, $n = 3$).

2.3.2 Gene Expression of Atrial and Ventricular Cardiac Markers in Day 30 iPSC-CMs

2.3.2.1 Gene Expression Analysis of Day 30 iPSC-CMs

Three batches of iPSCs were differentiated into atrial and ventricular cardiomyocytes and matured to 30 days after the initiation of differentiation. Atrial specification was achieved through the addition of 1.0 μ M RA at days 4 and 6 of differentiation. At day 30, the cells were harvested for RNA isolation and cDNA conversion. qPCR was performed on the three differentiation batches of iPSC-CMs.

2.3.2.2 Gene Expression of Structural Cardiac Markers in Day 30 iPSC-CMs

The expression of the following cardiac structural genes was assayed in ventricular and atrial iPSC-CMs: myosin heavy chain 7 (*MYH7*), myosin heavy chain 6 (*MYH6*), myosin light chain 7 (*MYL7*), myosin light chain 2 (*MYL2*) and natriuretic peptide A (*NPPA*). The *MYH7*

and *MYL2* genes respectively encode the ventricular isoforms of the myosin heavy and light chains. The *MYH6* and *MYL7* genes encode the atrial isoforms. The natriuretic peptide gene *NPPA* is expressed predominantly in atrial cardiomyocytes. The expression of structural cardiac genes in atrial and ventricular iPSC-CMs at 30 days after the initiation of cardiac differentiation is shown in *Figure 2.4*.

The mean expression level of *MYL7* was greater in the atrial iPSC-CMs but it was not statistically significant. The ventricular iPSC-CMs demonstrated significantly higher expression of *MYL2*. The atrial iPSC-CMs showed a large upregulation in *NPPA* expression. The atrial isoform of the myosin heavy chain, *MYH6*, was expressed significantly greater in the atrial cells and was accompanied with a downregulation in the ventricular isoform *MYH7*.

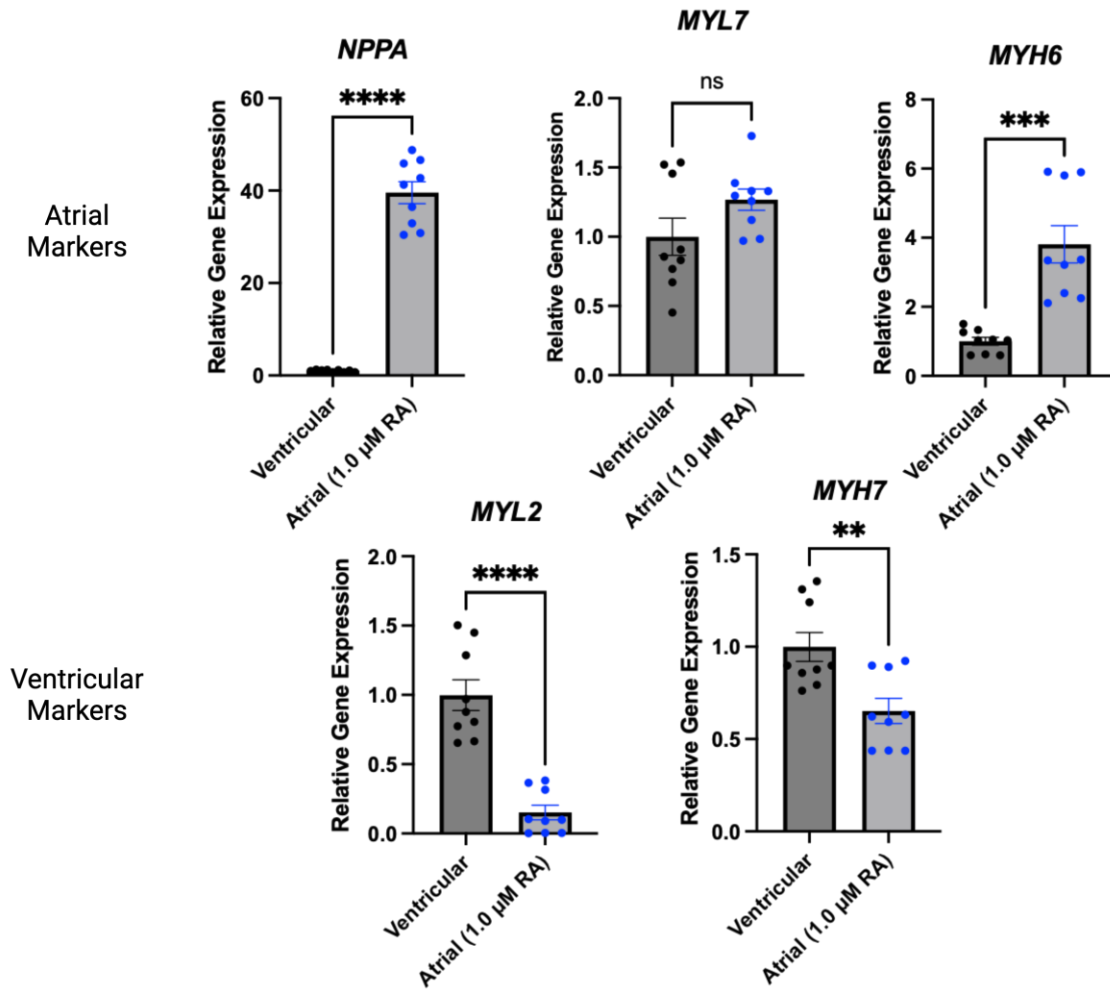


Figure 2.4: Gene Expression of Ventricular and Atrial Structural Cardiac Markers in iPSC-CMs (Day 30)

qPCRs were performed for the cardiac genes myosin heavy chain 7 (*MYH7*), myosin heavy chain 6 (*MYH6*), myosin light chain 7 (*MYL7*), myosin light chain 2 (*MYL2*), natriuretic peptide A (*NPPA*). Cells were either treated with no retinoic acid (Ventricular) or 1.0 μ M retinoic acid (Atrial) during cardiac differentiation. The expression levels of all the genes were normalised to *GAPDH* gene expression. Individual data points (technical replicates) and the mean expression level and SEM are shown in the graph for each condition. A Welch's t-test was used to ascertain significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ (N = 3, n = 9).

2.3.2.3 Gene Expression of Ion Channel Subunit Genes in Day 30 iPSC-CMs

The expression of the following ion channel subunit genes was assayed in ventricular and atrial iPSC-CMs: potassium voltage-gated channel subfamily A member 5 (*KCNA5*), potassium voltage-gated channel subfamily A member 4 (*KCNA4*), sodium voltage-gated channel alpha subunit 5 (*SCN5A*), potassium inwardly rectifying channel subfamily J member 3 (*KCNJ3*), potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*) and potassium voltage-gated channel subfamily H Member 2 (*KCNH2*). The expression levels of the ion channel subunit genes in ventricular and atrial iPSC-CMs are shown in Figure 2.5.

The atrial iPSC-CMs demonstrated a significant downregulation in *KCNA4* gene expression. The ventricular iPSC-CMs showed significantly greater levels of *KCNJ5* expression. Variable expression of *SCN5A* was observed in both the ventricular and atrial iPSC-CMs, with no significant difference seen between the conditions. A significant upregulation of *KCNJ3* expression was observed in the atrial iPSC-CMs. No significant difference was seen in *KCNA5* gene expression between the ventricular and atrial iPSC-CMs. Expression levels of *KCNH2* were slightly higher in the atrial iPSC-CMs, however this was not statistically significant.

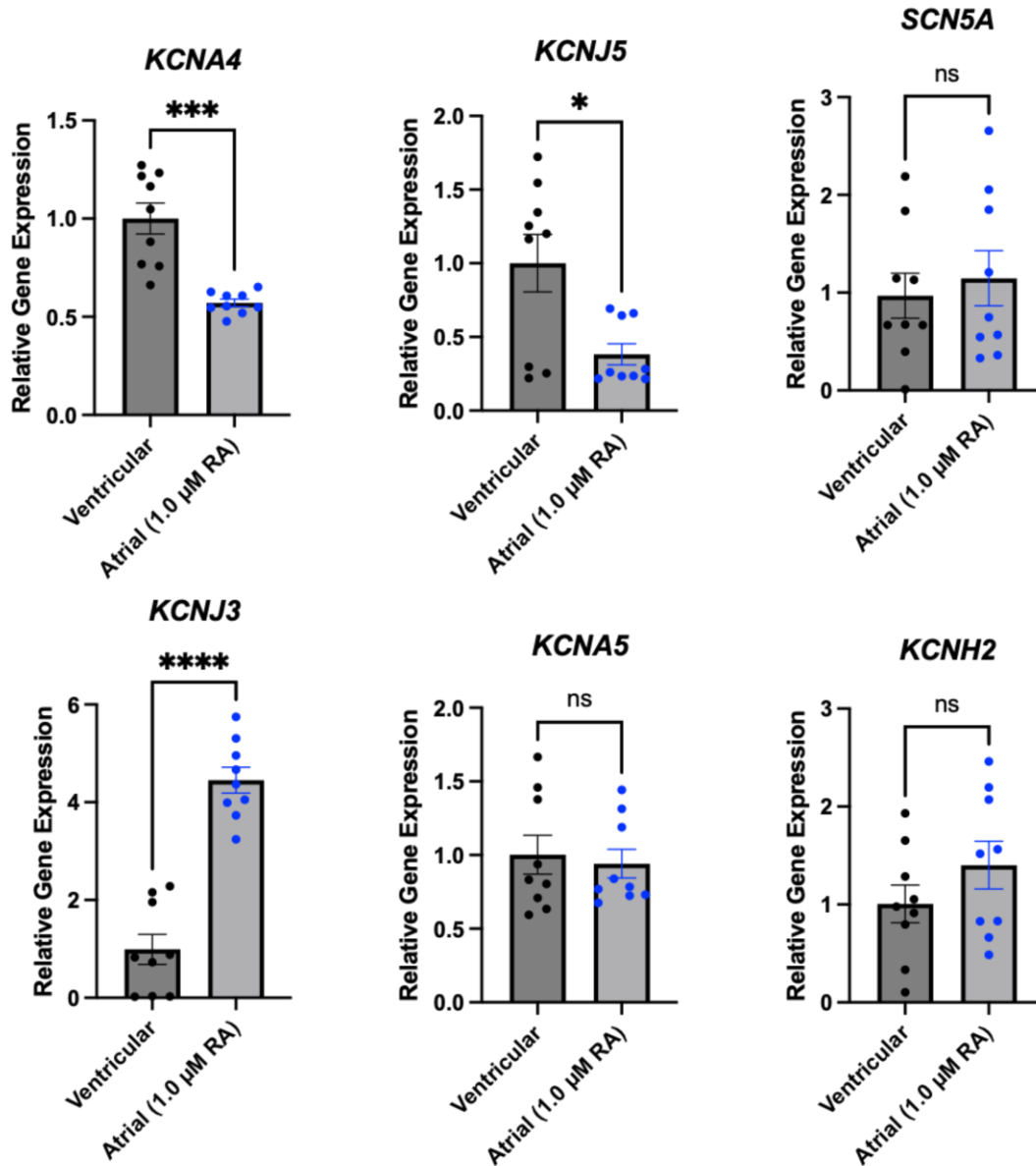


Figure 2.5: Gene Expression of Ion Channel Subunit Genes in Ventricular and Atrial iPSC-CMs (Day 30)

qPCRs were performed for the ion channel subunit genes: potassium voltage-gated channel subfamily A member 5 (*KCNA5*), potassium voltage-gated channel subfamily A member 4 (*KCNA4*), potassium inwardly rectifying channel subfamily J member 3 (*KCNJ3*), potassium inwardly rectifying channel subfamily J member 5 (*KCNJ5*), potassium voltage-gated channel subfamily H Member 2 (*KCNH2*), sodium voltage-gated channel alpha subunit 5 (*SCN5A*). Cells were either treated with no retinoic acid (Ventricular) or 1.0 μM retinoic acid (Atrial) during cardiac differentiation. The expression levels of all the genes were normalised to *GAPDH* gene expression. Individual data points (technical replicates), the mean expression level and SEM are shown in the graph for each condition. A Welch's t-test was used to test significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ (N = 3, n = 9).

2.3.3 Sarcomere Analysis of Ventricular and Atrial iPSC-CMs

iPSCs were differentiated into ventricular and atrial cardiomyocytes and matured until 30 days after the initiation of differentiation. The cells were stained for α -actinin 2, phalloidin (F-actin) and DAPI (Nuclear Marker). Images were taken at x63 magnification from three batches of atrial (1.0 μ M retinoic acid) and ventricular iPSC-CMs. An example of one of the images used for the analysis is shown in *Figure 2.6*. Sarcomere analysis was performed using the Z-line detection software described in Morris et al., 2020 to determine sarcomere length in the ventricular and atrial iPSC-CMs. α -actinin 2 identifies the z-lines, with the distance between two adjacent z-lines dictating the sarcomere length. F-actin is used to create a mesh, which can be used to mitigate inclusion of anomalous off-target staining in the analysis and provide more accurate identification of z-lines.

The mean sarcomere lengths and orientation order parameters (organisation) of the ventricular and atrial iPSC-CMs are shown in *Figure 2.7*. The sarcomere lengths of the ventricular and atrial iPSC-CMs were not significantly different, with the atrial cells showing a mean sarcomere length of approximately 2.01 μ m and the ventricular cells showing a mean length of 2.02 μ m. The ventricular and atrial iPSC-CMs showed similarly organised z-lines.

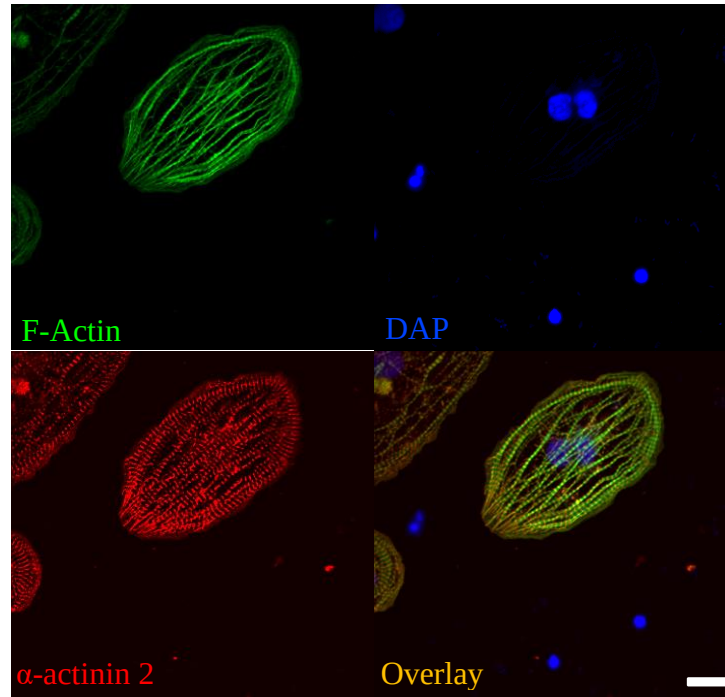


Figure 2.6: Example of Image Used for Sarcomere Analysis of Ventricular and Atrial iPSC-CMs

Atrial iPSC-CMs were fixed and stained for F-Actin (Top Left), DAPI (Top Right) and α -actinin 2 (Bottom Left). An overlay is shown on the bottom right. The scale bar is 20 μ m in length.

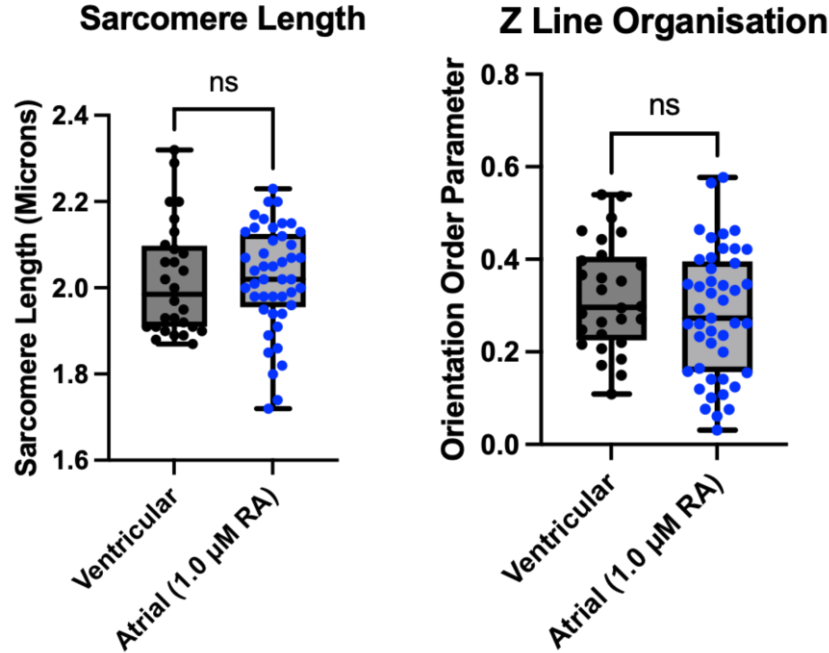


Figure 2.7: Sarcomere Lengths of Ventricular and Atrial iPSC-CMs

Images were taken from three differentiation batches of ventricular and atrial iPSC-CMs. The images were subsequently analysed using the Morris et al., (2020) Z-line detection script. The mean sarcomere lengths and Z-line organisation of the ventricular and atrial iPSC-CMs are plotted above. Ventricular N = 3, n = 28 images. Atrial N = 3, n = 45. A Welch's t-test was used to ascertain significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$.

2.3.4 Immunofluorescence Protein Expression of MLC-2v/2a in iPSC-CMs

iPSCs were differentiated into ventricular and atrial cardiomyocytes and matured until 30 days after the initiation of differentiation. The cells were co-stained for both the myosin regulatory light chain 2 ventricular isoform (MLC-2v) and the myosin regulatory light chain 2 atrial isoform (MLC-2a), as well as for DAPI (nuclear marker). Images were taken of both groups of cells to identify and count the number of cells which stained positive for MLC-2v, MLC-2a, or both markers. A representative image was taken at x10 magnification of the ventricular and atrial cells and is shown in *Figure 2.8*. 12 images of the ventricular and atrial iPSC-CMs were taken to determine the relative proportions of the cells expressing each or both of the protein isoforms. The number of cells in each image positive for each and both of the markers were counted, and the percentages of marker expression can be seen in *Figure 2.9*.

The atrial iPSC-CMs had a significantly greater percentage of cells demonstrating the MLC-2a+/MLC-2v- staining pattern than the ventricular cells, with respective means of 85 % and 15 %. The ventricular iPSC-CMs showed significantly greater co-expression of MLC-2a and MLC-2v than the atrial iPSC-CMs, with respective means of 23 % and 8 %. The mean percentage of cells demonstrating MLC-2a-/MLC-2v+ for the ventricular iPSC-CMs was 63 % and was significantly greater than expression in the atrial iPSC-CMs (mean = 6 %). Overall, the vast majority of the atrial cells were positive for MLC-2a staining (93 %). Approximately 86 % of the ventricular cells stained positive for MLC-2v, with a greater proportion of the cells co-expressing MLC-2a.

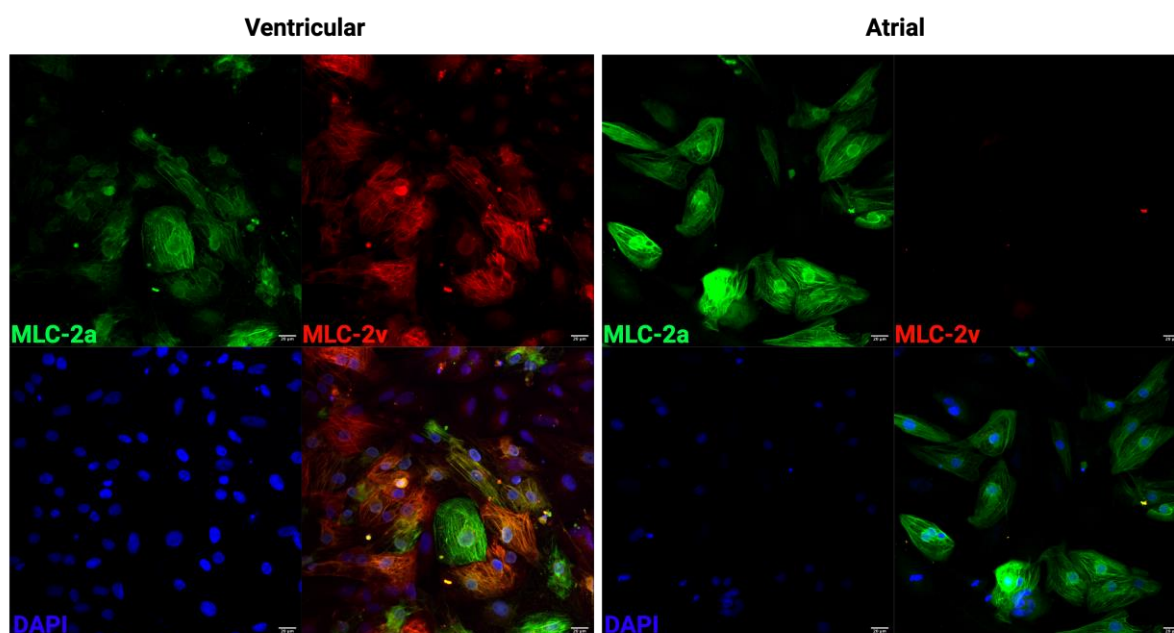


Figure 2.8: Representative Image of Ventricular and Atrial iPSC-CMs stained for MLC-2v/2a

Ventricular and Atrial iPSC-CMs were stained for MLC-2v (Top Right), MLC-2a (Top Left) and DAPI, (Bottom Left). Scale bar = 20 μm . Ventricular iPSC-CMs are shown on the left. Atrial iPSC-CMs are shown on the right.

Staining Pattern of MLC-2v/2a in iPSC-CM

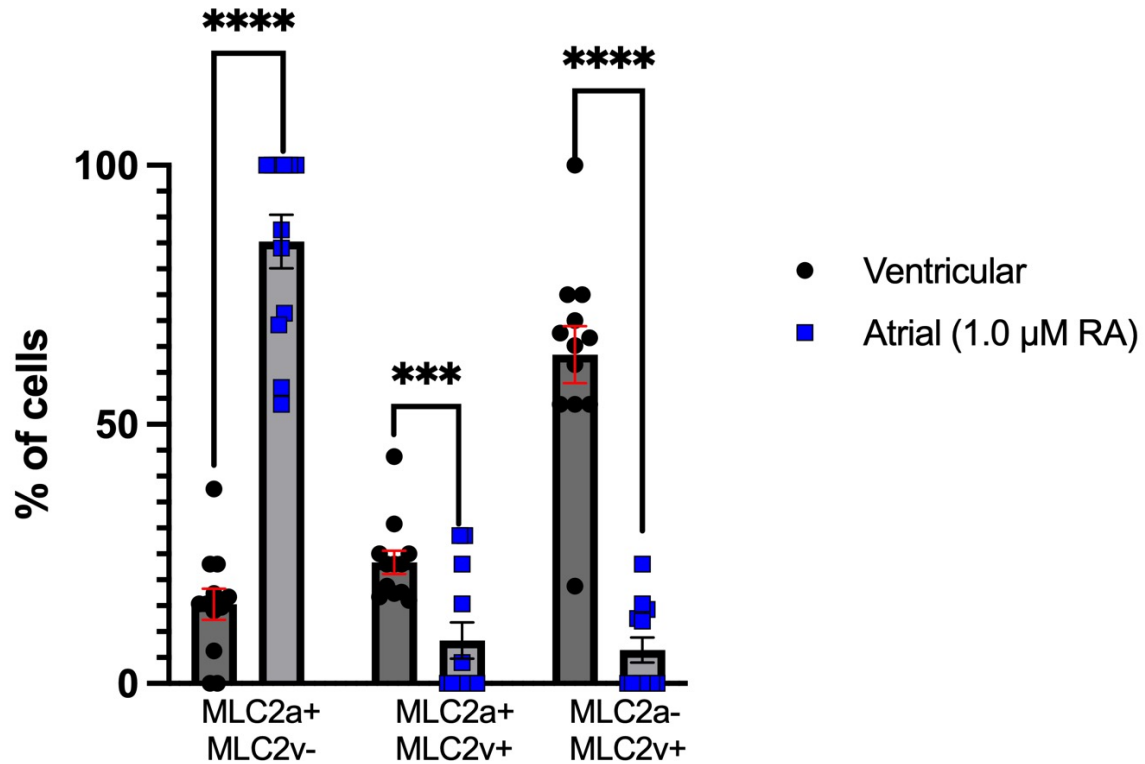


Figure 2.9: Relative Staining Patterns of MLC-2v and MLC-2a in iPSC-CMs

The percentage of cells positive for either or both isoforms of MLC-2v and MLC-2a, in images of ventricular (black) and atrial (blue) (1.0 μ M RA) iPSC-CMs. Individual data points (images assessed) and the mean percentage of cells expressing the marker pattern are shown in the graph for each condition. Multiple Welch's t-tests were used to ascertain significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ (N = 1, n = 12).

2.3.5 Western Blot Expression of MLC-2v

Three batches of ventricular and atrial iPSC-CMs were differentiated from iPSCs and matured to 30 days after the initiation of differentiation. The cells were spun down and protein samples were prepared according to the methodology outlined in 2.2.4. A western blot was performed for the ventricular specific myosin light chain 2 (MLC-2v). Images of the stained membranes for MLC-2v and GAPDH are shown in *Figure 2.10*. The ventricular iPSC-CMs showed detectable expression of MLC-2v, whilst the atrial iPSC-CMs did not demonstrate detectable protein expression.

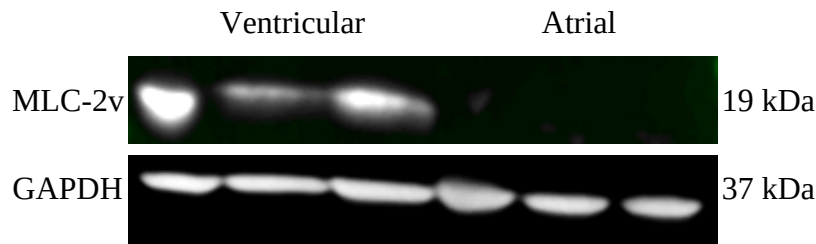


Figure 2. 10: Protein Expression of MLC-2v and GAPDH in Ventricular and Atrial iPSC-CMs

3 samples of ventricular and atrial iPSC-CMs (N =3) were run on a polyacrylamide gel and blotted for MLC-2v (top) and GAPDH (bottom). The ventricular iPSC-CMs showed expression of MLC-2v. No expression was observed from the atrial iPSC-CMs.

2.3.6 Action Potentials of Ventricular and Atrial iPSC-CMs

A single batch of ventricular and atrial iPSC-CMs was differentiated and matured to 30 days after the initiation of differentiation. The cells were split onto coverslips. Stimulated action potentials were derived by current clamping the cells. The action potentials were analysed for upstroke velocity, amplitude and duration. Maximum diastolic potential was recorded prior to holding the cells at -75 mV and stimulating action potentials. Analysis of the stimulated action potentials is shown in *Figure 2.11*. Action potential morphology was analysed to determine how ventricular or atrial like the action potentials were. This was performed by calculating the APD30-40/APD70-80, as described in Ma et al., (2011). Values > 1.5 represent a more ventricular like action potential morphology, whilst values less than 1.5 indicate a more atrial like phenotype. The results of this analysis, as well as representative traces from the ventricular and atrial iPSC-CMs, are shown in *Figure 2.12*.

The maximum diastolic potential was significantly more negative in the ventricular iPSC-CMs. Therefore, the mean current needed to be injected to hyperpolarise the cells to -75 mV was 34 pA for the ventricular iPSC-CMs and 38 pA for the atrial iPSC-CMs. The median upstroke velocity of the ventricular iPSC-CMs was significantly greater than the atrial cells, with respective medians of 124 and 107 mV/ms. The cells showed no significant differences in action potential amplitude. The action potential durations at 30, 50, 70 and 90 %

repolarisation were significantly prolonged in the ventricular iPSC-CMs. The action potential morphology values were significantly greater in the ventricular iPSC-CMs, with only one morphology value of the atrial iPSC-CMs being greater than the ‘ventricular like’ 1.5 threshold. The action potentials derived from the ventricular iPSC-CMs showed greater variability in upstroke velocity, amplitude, duration and APD morphology.

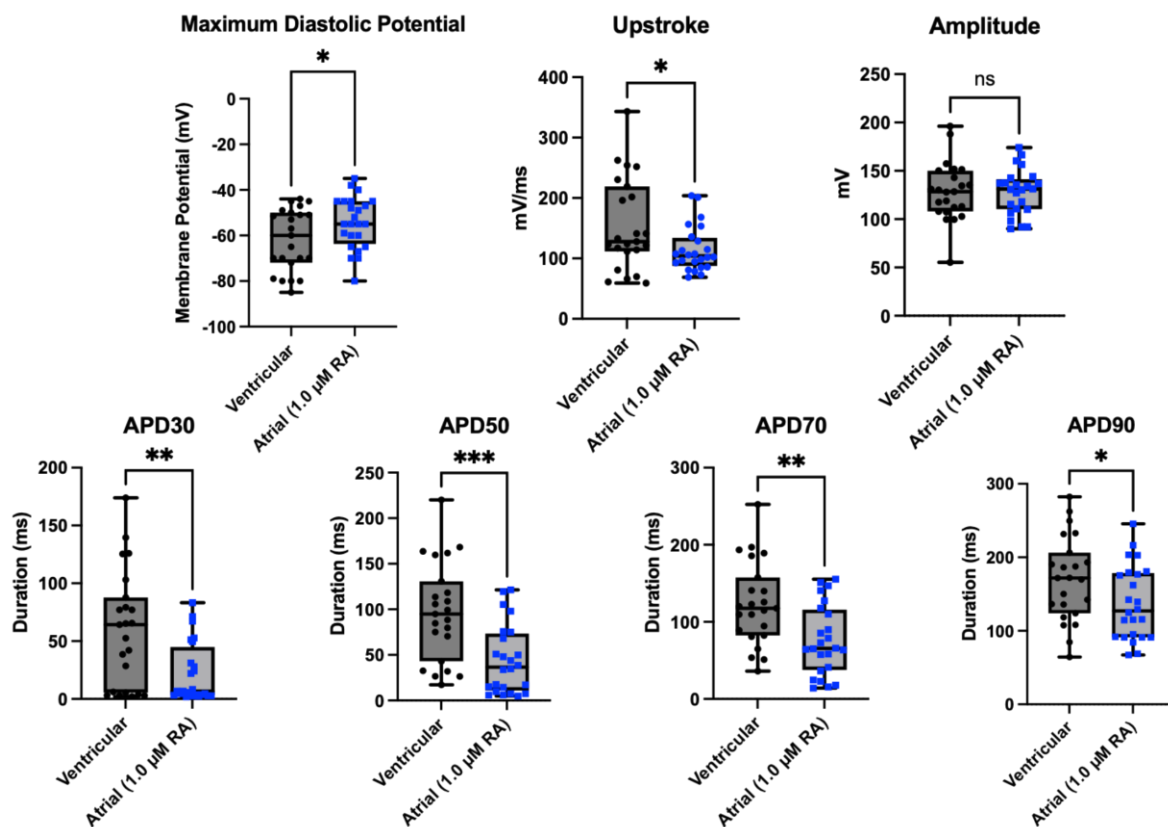


Figure 2.11: Analysis of Stimulated Action Potentials from Ventricular and Atrial iPSC-CMs

Action potentials were stimulated from ventricular and atrial iPSC-CMs. Action potentials were stimulated at a frequency of 1 Hz using a 1 nA depolarising current. Maximum diastolic potential was recorded prior to holding the cells at -75 mV. Action potentials were analysed for upstroke velocity, amplitude, and duration at 30, 50, 70 and 90 % repolarisation (APD 30, APD50, APD70, APD90). A Welch's t-test was used to ascertain significance. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$. Ventricular N = 1, n = 23 cells. Atrial N = 1, n = 24.

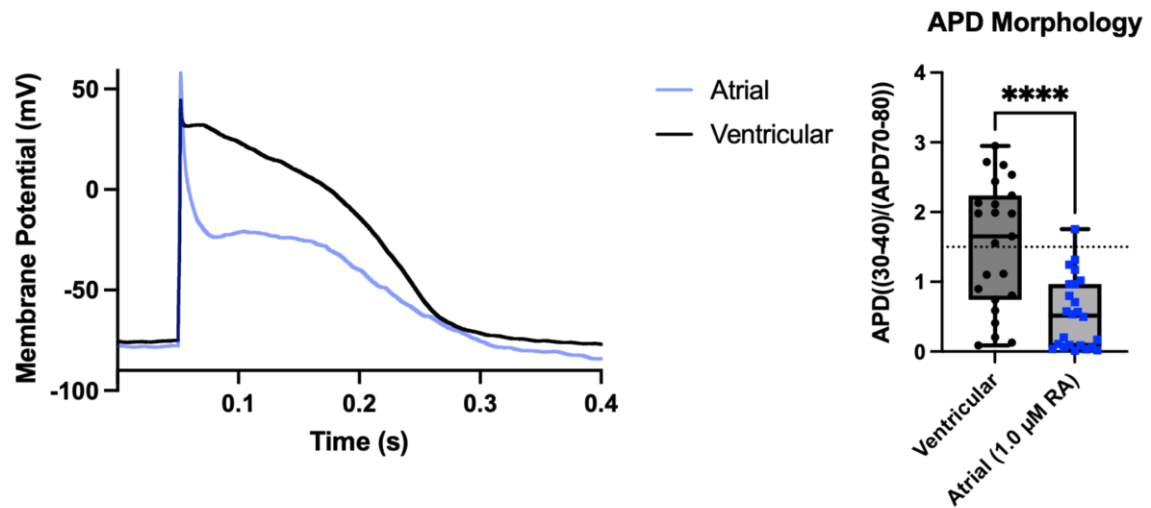


Figure 2.12: APD Morphology of Ventricular and Atrial iPSC-CMs

Action potentials were stimulated from ventricular and atrial iPSC-CMs. Action potentials were stimulated at a frequency of 1 Hz. A representative ventricular and atrial action potential is shown on the left. The APD_{30-40}/APD_{70-80} was calculated using the APD values for each individual cell. A value > 1.5 indicates a more ventricular like action potential shape, whilst a value < 1.5 indicates an atrial or nodal like action potential shape. Ventricular $N = 1$, $n = 23$ cells. Atrial $N = 1$, $n = 24$.

2.4 Discussion

2.4.1 Gene Expression of Atrial and Ventricular Markers in iPSC-CMs

2.4.1.1 *Gene Expression of Atrial and Ventricular Markers in iPSC-CMs at Day 20*

Assessing the ways in which TTNtv could increase the risk of AF, requires the generation of an atrial specific cardiomyocyte model. Manipulation of the retinoid signalling pathway has proven useful in the generation of compartment specific iPSC-CMs. During vertebrate development, retinol, also known as vitamin A, is converted into retinaldehyde and subsequently retinoic acid in the retinoid signalling pathway (Wiesinger et al., 2021).

Following this, retinoic acid is translocated to the nucleus where it upregulates expression of several genes including those that encode the transcription factors COUP-TFI and COUP-TFII (Dohn et al., 2019). These transcription factors bind to regulatory regions of DNA and promote expression of atrial specific genes such as the ion channel subunit genes *KCNJ3* and *KCNA5*. As no established protocol for atrial differentiation existed in the laboratory, prior to starting the project, optimisation of a protocol was necessary. A preliminary experiment investigating the effect 0.5 and 1.0 μM retinoic acid treatment had on the atrialisation of iPSC-CMs in the KOLC2 iPSC line was performed.

As previously described in 2.3.1, the ventricular and atrial (1.0 μM RA) iPSC-CMs showed the expected tissue specific expression of the myosin light chain genes, *MYL7* and *MYL2*.

However, gene expression of the atrial and ventricular heavy chain isoforms, *MYH6* and *MYH7*, was less distinct. Surprisingly the atrial (0.5 and 1.0 μM RA treated) cells showed higher expression of *MYH7* than their ventricular counterparts and only minimally more expression of *MYH6*. The unexpected expression levels of the compartment specific myosin heavy chain isoforms may have been due to the relative immaturity of the cells. It is convention, to mature iPSC-CMs to 30 days after the initiation of differentiation prior to the use of them in experiments. Maturing the cells for longer than 20 days would likely induce

tissue specific expression of the myosin heavy chain isoforms in the atrial and ventricular cells. Overall, the cells that were treated with 1.0 μ M retinoic acid showed greater expression of the atrial specific gene markers *MYH6* and *MYL7* than those treated with 0.5 μ M.

Reassuringly, gene expression of *TTN* was similar in both the ventricular (no RA) and atrial (1.0 μ M RA) iPSC-CMs. Gene expression of *ACTN2* decreased with increased retinoic acid treatment. Retinoic acid is commonly used in non-myocyte differentiation protocols, such as cardiac fibroblast differentiation (Zhang et al., 2019). Therefore, the populations of cells treated with retinoic acid may be more heterogenous and contain a larger proportion of non-myocyte cells with diminished *ACTN2* expression.

Expression of genes encoding ion channel subunits were assessed in the ventricular and atrial iPSC-CMs. The majority of the genes investigated encoded voltage gated potassium ion channel subunits, which are broadly upregulated in atrial iPSC-CMs and are responsible for the shortened plateau and duration of the atrial action potential. The atrial iPSC-CMs (1.0 μ M RA) showed the expected tissue specific expression of *KCNJ3* and *KCNA4*. However, expression of the atrial specific ion channel subunit genes *KCNA5* and *KCNJ5* was higher in the ventricular iPSC-CMs. There was no detectable expression of *SCN5A* in either the ventricular or atrial iPSC-CMs. This was likely due to the immaturity of the cells at 20 days after the initiation of cardiac differentiation.

These preliminary experiments were performed to determine the optimum concentration of retinoic acid to use in future differentiations. Because RNA was derived from only a single differentiation batch of ventricular and atrial iPSC-CMs, it is difficult to confidently deduce the effects 0.5 and 1.0 μ M retinoic acid had on the expression of structural and ion channel encoding cardiac genes. The cells derived using 1.0 μ M RA showed high expression of the atrial specific genes *MYH6*, *MYL7* and *KCNJ3*. For this reason, 1.0 μ M RA was used in future atrial cardiomyocyte differentiations.

2.4.1.2 Expression of Atrial and Ventricular Markers in iPSC-CMs at Day 30

The ventricular and atrial iPSC-CMs broadly showed tissue specific expression of structural cardiac genes at 30 days after differentiation. Although showing higher mean gene expression, *MYL7* was not significantly upregulated in the atrial iPSC-CMs. This is in contrast to the immunofluorescence data where the protein encoded by *MYL7* (MLC-2a) showed much greater expression in the atrial iPSC-CMs. The reason for this is unclear. It is possible that performing qPCR for *MYL7* on a greater number of differentiation batches may reveal a statistically significant upregulation in the atrial cells.

The atrial iPSC-CMs showed the expected upregulation of *KCNJ3* and downregulation of *KCNA4*. Unexpectedly, there was no significant difference in *KCNA5* expression between the atrial and ventricular iPSC-CMs. The *KCNA5* gene encodes the K_v1.5 channel which is responsible for the ultrarapid delayed rectifier K⁺ current (I_{Kur}). The I_{Kur} current is upregulated in the atria and contributes towards the shortened atrial action potential (Christophersen et al., 2013). Previous studies, including Argenziano et al., (2018), have demonstrated robust increases in *KCNA5* expression in RA derived atrial iPSC-CMs. More differentiation batches may be required to uncover statistically significant differences in *KCNA5* expression. The ventricular iPSC-CMs showed significantly greater expression of *KCNJ5* which is predominantly expressed in the atria. Confounding expression levels of the ion channel subunit genes in the atrial iPSC-CMs may be caused by a lack of cell maturity. Furthermore, it is important to note that the gene expression levels determined may not necessarily equate to the amount of functional protein available in the cells. Both the atrial and ventricular iPSC-CMs demonstrated variable expression levels of *SCN5A* at day 30 of maturation. This contrasts with the day 20 iPSC-CMs where *SCN5A* was undetectable and is concordant with previous studies investigating *SCN5A* expression during iPSC-CMs maturation (Lemoine et al., 2017).

2.4.2 Sarcomere Analysis of Ventricular and Atrial iPSC-CMs

There was no significant difference in sarcomere length or z-line organisation between the ventricular and atrial iPSC-CMs. This is consistent with previous studies that have measured sarcomere lengths in cardiomyocytes derived from both compartments of the heart (Piroddi et al., 2007). The sarcomere lengths measured in the iPSC-CMs were within the range of lengths that have been reported for primary human cardiomyocytes (1.9 to 2.8 μm) (McDonald et al., 2020) (Bird et al., 2003). Previous studies assessing sarcomere length in iPSC-CMs have shown variable sarcomere lengths between 1.6 and 2.3 μm (Lemcke et al., 2020) (Skorska et al., 2022) (Mills et al., 2017). The mean lengths measured in both the ventricular and atrial iPSC-CMs were within the range of reported sarcomere lengths. Published sarcomere lengths are measured using a variety of techniques, algorithms, and equipment. This ultimately causes a large range in reported values. Furthermore, the differentiation protocol and stem cell line used will also likely affect the sarcomere lengths of the cells derived.

2.4.3 Protein Expression of MLC-2a/2v Isoforms in iPSC-CMs

The atrial iPSC-CMs showed significantly greater expression of the atrial isoform of the myosin regulatory light chain MLC-2a. Approximately half of the images taken showed cells solely expressing MLC-2a. The percentages of atrial cells demonstrating MLC-2a+/MLC-2v- staining was comparable to those found in Cyganek et al., (2018) (~ 90%). The ventricular iPSC-CMs demonstrated significantly greater co-expression of MLC-2a and MLC-2v, indicating that the atrialisation process not only increases MLC-2a expression but decreases expression of MLC-2v. Approximately 25 % of the ventricular iPSC-CMs showed MLC-2a+/MLC-2v- staining. This was not unexpected as previous studies using similar cardiac differentiation protocols have been shown to produce a variety of ventricular, atrial and nodal

iPSC-CMs (Bhattacharya et al., 2014) (Bizy et al., 2013) (Cordeiro et al., 2013). Co-expression of MLC-2a and MLC-2v is seen *in vivo* during ventricular chamber specification, with loss of MLC-2a expression occurring during ventricular cardiomyocyte maturation (Kubalak et al., 1970). Therefore, it is possible that the cells co-expressing both markers are either terminally differentiated but immature cardiomyocytes or are still undergoing ventricular transition and maturation. The results of the MLC-2a/MLC-2v immunostaining are broadly consistent with the gene analysis of *MYL7* and *MYL2*. The atrial cells showed minimal expression of the ventricular isoform of the gene, whilst the ventricular cells showed robust expression of *MYL7* expression. An alternative and more quantitative approach to measuring MLC-2a/MLC-2v expression would be to perform flow cytometry on cells stained with the 2 markers. This would have given a better measurement on the exact percentages of cells expressing the markers, but it is limited by its inability to assess correct staining pattern. Western blot analysis of MLC-2v revealed no expression of MLC-2v in the atrial iPSC-CMs, but expression in the ventricular cells. This is consistent with the expression of the *MYL7* (MLC-2v) gene, which showed minimal expression in the atrial iPSC-CMs.

2.4.4 Action Potentials of Ventricular and Atrial iPSC-CMs

The upstroke velocities of the ventricular and atrial iPSC-CMs (100 mV/mS) were significantly slower than those of primary human cardiomyocytes (200-300 mV/ms) but were consistent with other 2D iPSC-CMs models (Lemoine et al., 2017) (Sottas et al., 2018). Slower upstroke velocities in iPSC-CMs occur due to a reduction in the I_{K1} current and expression of foetal *SCN5A* isoforms (Veerman et al., 2017) (Goversen et al., 2018). The ventricular iPSC-CMs showed a significant increase in upstroke velocity relative to the atrial cells. Primary ventricular cardiomyocytes have greater upstroke velocities than their atrial counterparts, with respective values of approximately 300-400 and 150-300 mV/ms (Amos et al., 1996) (Wang et al., 2003). This is predominantly due to differences in resting membrane

potential and sodium channel availability, but also due to channel isoform expression. Action potentials were stimulated from ventricular and atrial iPSC-CMs held at -75 mV. Therefore, it is likely that the increased upstroke velocities observed from the ventricular iPSC-CMs was due to differences in the abundance or isoform expression of inward sodium channels. The action potential amplitudes of the atrial and ventricular iPSC-CMs were approximately 130 mV and broadly similar to the amplitudes recorded in isolated primary cardiomyocytes. The action potential durations were significantly longer in the ventricular iPSC-CMs at 30, 50, 70 and 90 % repolarisation. Large variability was seen in the APD30 and 50 of both the ventricular and atrial iPSC-CMs due to presence or absence of the transient outward potassium current. The difference in duration was assessed from 10-90 % repolarisation. The APDs were significantly longer in the ventricular iPSC-CMs from 20 to 90 % repolarisation. This is consistent with previous studies looking at the relative durations of action potentials recorded from atrial and ventricular primary cardiac myocytes. The atrial iPSC-CMs needed 38 pA of current to bring the membrane potential down to -75 mV, while the ventricular iPSC-CMs needed just 34 pA. Therefore, the shortened APDs of the atrial iPSC-CMs may be due to the greater hyperpolarising current injected into the cells and not compartment specificity. This is a limitation with this approach.

The maximum diastolic potential (MDP) of the ventricular and atrial iPSC-CMs was considerably more positive than the resting membrane potentials recorded in primary atrial (-80 mV) and ventricular (-90 mV) cardiomyocytes and is a sign of the immaturity of the cells (Goversen et al., 2018). The average MDP was significantly more negative in the ventricular iPSC-CMs. The APD30-40/APD70-80 has previously been used to determine how atrial or ventricular like the shape of the action potential is in iPSC-CMs (Ma et al., 2013) (Doss et al., (2012) (Argenziano et al., 2018). A cut off is normally applied with a value greater or less than 1.5 indicating respectively a ventricular or atrial like action potential. All but one of the

values calculated for the atrial iPSC-CMs was below 1.5, suggesting the cells broadly demonstrate atrial like action potentials. Approximately 40 % of the ventricular iPSC-CMs demonstrated values below 1.5. This is consistent with the MLC staining patterns of the ventricular iPSC-CMs, which showed approximately 40 % of the cells staining positive for MLC-2a. This method of assigning a chamber specific phenotype through an arbitrary cut-off in action potential morphology has been strongly criticized (Kane et al., 2016). Critics have denoted that the large variability of action potential durations means that different morphologies/durations can exist within the continuum without designating chamber specificity and that action potential characteristics are too heavily influenced by cell density to be a chamber marker. Therefore, action potential morphology of iPSC-CMs should always be interpreted within a context where additional structural and genetic markers of chamber specificity have been measured.

2.4.5 Conclusion

iPSCs were treated with retinoic acid during cardiac differentiation to promote atrial specification. Treating the cells with 1.0 μ M retinoic acid caused the upregulation of atrial specific structural and ion channel subunit genes. Furthermore, it increased protein expression of the atrial specific myosin light chain and decreased expression of the ventricular isoform. The cells differentiated using 1.0 μ M retinoic acid demonstrated atrial like action potentials upon stimulation and were broadly more homogenous than their ventricular counterparts when considering electrophysiology and myosin light chain isoform expression. Standard cardiac differentiation methods putatively yield predominantly ventricular like iPSC-CMs. The cells produced without the addition of retinoic acid showed greater expression of ventricular specific structural and ion channel subunit genes. However, analysis of action potentials and myosin light chain expression indicated that the population

of cells generated consisted of a mixture of ventricular and atrial like cardiomyocytes. The atrial iPSC-CMs differentiated recapitulated some of the genetic, structural and electrophysiological characteristics of primary human atrial cardiomyocytes and were consequently considered an adequate model in which to study familial AF.

CHAPTER 3: Generation of a human iPSC derived Cardiomyocyte/Fibroblast Engineered Heart Tissue Model

The following has been submitted to the *NC3Rs F1000Research Gateway* as a method article.

Contractility analysis on videos of EHTs was performed by Mr Jonas Euchner (University of Birmingham). Author contributions are listed on the next page.

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Roles: Formal Analysis, Methodology, Software, Writing – Review & Editing

Amar J Azad

Roles: Conceptualization, Methodology, Supervision, Writing – Review & Editing

Nguyen T N Vo

Roles: Methodology, Resources, Writing – Review & Editing

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Roles: Supervision, Writing – Review & Editing

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Roles: Conceptualization, Funding Acquisition, Project Administration, Resources, Supervision, Writing – Review & Editing

ABSTRACT

Animal models have proven integral to broadening our understanding of complex cardiac diseases but have been hampered by significant species-dependent differences in cellular physiology. Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) have shown great promise in the modelling of cardiac diseases despite limitations in functional and structural maturity. 3D stem cell derived cardiac models represent a step towards mimicking the intricate microenvironment present in the heart as an *in vitro* model. Incorporation of non-myocyte cell types, such as cardiac fibroblasts, into engineered heart tissue models (EHTs) can help better recapitulate the cell to cell and cell to matrix interactions present in the human myocardium. Integration of human induced pluripotent stem cell derived cardiac fibroblasts (iPSC-CFs) and iPSC-CMs into EHT models enables the generation of a genetically homogeneous modelling system capable of exploring the abstruse structural and electrophysiological interplay present in cardiac pathophysiology. Furthermore, the construction of more physiologically relevant 3D cardiac models offers great potential in the replacement of animals in heart disease research. Here we describe efficient and reproducible protocols for the differentiation of iPSC-CMs and iPSC-CFs and their subsequent assimilation into EHTs. The resultant EHT consists of longitudinally arranged iPSC-CMs, incorporated alongside iPSC-CFs. EHTs with both iPSC-CMs and iPSC-CFs exhibit slower beating frequencies and enhanced contractile force compared to those composed of iPSC-CMs alone. The modified protocol may help better characterise the interplay between different cell types in the myocardium and their contribution to structural remodelling and cardiac fibrosis.

Keywords

Engineered Heart Tissue (EHT), cardiac model, induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs), induced pluripotent stem cell derived fibroblasts (iPSC-CF), cardiac disease modelling, cardiac fibrosis

Scientific Benefits

- Generation of a more physiologically relevant human cardiac model
- Utility as a genetically homogenous system for disease modelling of cardiac arrhythmia, heart disease and cardiomyopathy
- Capacity in exploring cellular dynamics integral to cardiac fibrosis

3Rs Benefits

- Engineered heart tissue models can replace the use of small animal models of cardiac arrhythmia, cardiomyopathy and heart failure
- Acquiring data from the small animal models used in cardiovascular research often requires the animal to undergo multiple moderate severity procedures which require repeated anaesthesia. Such procedures can include electrocardiograms and mini pump/telemetry implantation
- Many of the pathogenic variants modelled in mouse lines cause chronic and severe illness
- As mouse physiology is vastly different to human hearts, insights from mouse models cannot always be extrapolated to human hearts. In contrast, engineered heart tissue models use human cells with human physiology

Practical Benefits

- The relatively low cost in comparison to mouse models
- Induced pluripotent stem cells are relatively easier to genetically manipulate than *in vivo* models
- Ability to generate healthy human quiescent cardiac fibroblasts
- Reproducible methods for the derivation of cardiomyocytes and cardiac fibroblasts from iPSCs
- Accessibility of cardiac cells with a consistent genome

Current Applications

- Disease modelling of cardiac arrhythmia, cardiomyopathy and heart failure
- Exploring physiological interplay between cardiac fibroblasts and cardiomyocytes

Potential Applications

- Low throughput drug screening platform for cardiotoxicity
- Use as a human model to investigate the reversibility of cardiac fibrosis

3.1 Introduction

Limited availability of healthy human heart tissue combined with its inherent nature as a non-proliferative tissue type has meant that heart disease researchers have historically been reliant on *in vivo* models. Large animal models used in heart disease research, for example dogs, pigs, and sheep, are broadly analogous to humans in cardiac physiology and anatomy but are heavily restricted by cost, throughput, and the sentiment of the general public (Camacho et al., 2016). Small animal models commonly used in cardiovascular research, such as the mouse and zebrafish, offer a cheaper but drastically less physiological and anatomically similar alternative. Zebrafish offer distinct advantages including optical transparency and rapid development but are limited in their capacity as a cardiac model by their two-chambered heart and temperature dependent action potential (Vornanen and Hassinen, 2016). Significant differences in the intracellular electrophysiology of mouse cardiomyocytes, caused by contrasting expression and activation of the delayed rectifier and transient outward K^+ currents as well as the voltage-gated sodium and calcium channels, are broadly illustrated in their comparatively rapid heart rate of between 500-700 bpm (Xu et al., 1999) (Niwa and Nerbonne, 2010) (Blehschmidt et al., 2008). Consequently, the modelling of cardiac diseases such as arrhythmia, cardiomyopathy and heart failure, which often manifest through concomitant electrical and structural remodelling, is often hampered in mice due to distinct species dependent differences.

The co-development of human induced pluripotent stem cell (iPSC) technology and CRISPR-Cas 9 genome editing has provided a new type of genetically modified disease model, based on human cells (Ma et al., 2018) (Mosqueira et al., 2018). The utility of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) has caused a shift in the approach many cardiovascular researchers take to cardiac disease modelling, with many groups opting to replace or supplement the use of small animal models with comparatively cheaper stem cell-

based models (Cumberland et al., 2022). Consequently, iPSC-CMs have been used to successfully model pathogenic variants that predispose individuals to cardiac diseases, including arrhythmia and cardiomyopathy (Lin et al., 2015) (Goktas Sahoglu et al., 2022) (Jung et al., 2022) (Reyat et al., 2020) (Schulz et al., 2023).

The incorporation of iPSC-CMs into cardiovascular disease modelling has however not come without difficulty. Early attempts at differentiating cardiomyocytes produced variable and electrophysiologically immature cells with depolarized diastolic membrane potentials, slow action potential upstroke velocities and large pacemaker currents (Knollmann, 2013).

Optimisation and development of differentiation protocols enabled the derivation of chamber specific cardiomyocytes to explore diverse disease aetiologies but offered marginal advancements in variability and maturity (Jeziorowska et al., 2017) (Devalla et al., 2015).

Accordingly, innovative maturation strategies were developed to improve the maturity of the cells produced. Current methods include: micropatterning, metabolic maturation, electrical stimulation, soft-substrate culture, co-culture, and engineered 3D models (Jimenez-Vazquez et al., 2022) (Feyen et al., 2020) (Yoshida et al., 2018) (Tzatzalos et al., 2016) (Machiraju and Greenway, 2019).

With greater electrophysiological, metabolic, structural, and functional maturity, 3D cardiac models offer greater potential in understanding the nuances of complex cardiac diseases such as heart failure and cardiomyopathy (Correia et al., 2018) (Shadrin et al., 2017) (Ulmer et al., 2018) (Vučković et al., 2022). Furthermore, the concurrent ease by which contractile force and transmembrane potential can be monitored in 3D cardiac models presents great potential for their future incorporation into *in vitro* cardiotoxicity studies (Gintant et al., 2019). A number of 3D cardiac models have been generated to examine cardiac physiology in disease including, but not limited to: two-post engineered heart tissues (EHTs), ring shaped engineered heart muscle (EHM), cardiac patches, cardiac micro-, and biowires, and

Novoheart (Tiburcy et al., 2017) (Ronaldson-Bouchard et al., 2018) (Zhang et al., 2013) (Thavandiran et al., 2013) (Zhao et al., 2019). Although differing substantially in size and shape, all the 3D cardiac models listed above attempt to better recapitulate the cell to cell and cell to matrix interactions present in the myocardium and encourage the longitudinal alignment of cardiomyocytes upon a scaffold (Smith et al., 2017).

Despite occupying the majority of the volume of the mammalian heart, cardiac myocytes are estimated to account for less than 50% of the total cell number (Zhou and Pu, 2016). Major non-myocyte cell types in the heart include cardiac fibroblasts and endothelial cells as well as ancillary populations of immune cells and autonomic neurones. Therefore, the incorporation of non-myocyte cell populations into cardiac modelling systems should facilitate the structural, metabolic, and electrophysiological maturity of iPSC-CMs and aid in generating a more physiologically relevant microenvironment (Yoshida et al., 2018) (Kim et al., 2010).

Up to now, cell types commonly incorporated into 3D cardiac models include endothelial cells, mesenchymal stem cells, and cardiac fibroblasts (Tadano et al., 2021) (Tulloch et al., 2011) (Zhang et al., 2017).

Cardiac fibroblasts are a common cell type in the human ventricles (circa 20 % by number, [Pinto et al., 2016]) and are integral to the architecture, alignment, and electromechanical properties of the myocardium in health and disease. Quiescent or inactive induced pluripotent stem cell derived cardiac fibroblasts (iPSC-CF) can be derived from iPSCs and used effectively as a stand-alone model of cardiac fibrosis (Zhang et al., 2019). The activation of the quiescent cardiac fibroblast into a myofibroblast occurs prior to and during the development of cardiac fibrosis and is often difficult to prevent *in vitro* since the cells cultured on plastic dishes are subject to a Young's modulus up to a million times stiffer than the native myocardium (Landry et al., 2019) (Acevedo-Acevedo and Crone, 2015). This pathological transition is substantially stimulated by the profibrotic signalling cytokine TGF-

$\beta 1$. Suppression of TGF- $\beta 1$ can be achieved *in vitro* using small molecule inhibitors such as SB431542 and can be used on *in vitro* models to control the activation status of quiescent cardiac fibroblasts (Law and Carver, 2013).

An amalgamated 3D cardiac model consisting of iPSC-CF and iPSC-CMs presents the opportunity for the generation of genetically homogenous (isogenic) systems capable of investigating the pathophysiological interplay present in cardiac diseases such as cardiomyopathy and arrhythmia. Here we describe a modified protocol for the generation and incorporation of quiescent iPSC-CF into an engineered heart tissue model.

In this study we describe efficient protocols for the differentiation of iPSC-CMs and quiescent cardiac fibroblasts. Methods are outlined detailing the incorporation of these cells into EHTs with improved contractile function and tissue compaction and potential use exploring the pathophysiological interplay between iPSC-CMs and iPSC-CF in cardiac fibrosis. In the UK, 51,427 procedures were carried out on mice in 2021 for research on the cardiovascular, blood and lymphatic system (Home Office Report on Annual Statistics of Scientific Procedures on Living Animals Great Britain 2021). Assuming that 1 % of these mice were used to study cardiac arrhythmia, heart failure or cardiomyopathy, use of the model proposed could lead to the direct replacement of 500 mice per year in the UK alone.

3.2 Materials and Methods

3.2.1 Reagents for iPSC Culture and Differentiation

Table 3.1: Reagents for iPSC Culture and Differentiation

Reagent and Preparation	Company and Catalogue Number
Kolfc2 (WTSli018-B)	https://ebisc.org/WTSli018-A
mTeSR™ Plus	StemCell Technologies, 100-0276
DPBS, no calcium, no magnesium	Thermo Fisher Scientific, 14190144
Corning™ Costar™ 6-well Clear TC-treated	Fisher Scientific, 10578911
Corning 25 cm² cell culture flask TC-treated rectangular canted neck	Sigma-Aldrich, CLS430639-200EA
Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Thermo Fisher Scientific, A1413201
TrypLE™ Express Enzyme (1X), phenol red	Thermo Fisher Scientific, 12605010
TrypLE™ Select Enzyme (10X), no phenol red	Thermo Fisher Scientific, A1217701
Rock Inhibitor Y-27632 (Dihydrochloride) (Reconstituted in PBS to 10 mM and stored at -20 °C in 50 µl aliquots)	StemCell Technologies, 72304
StemPro™-34 SFM (1X)	Thermo Fisher Scientific, 10639011
L-Glutamine (200 mM)	Thermo Fisher Scientific, 25030081
Recombinant Human BMP-4 Protein (Reconstituted in 4 mM HCl (0.1 % BSA) to 50 µg/mL and stored at -80 °C in 5 µl aliquots)	R&D Systems, 314-BP-010/CF
Human Activin A Recombinant Protein (Reconstituted in PBS (0.1 % BSA) to 10 µg/mL and stored at -20 °C in 20 µl aliquots)	Thermo Fisher Scientific, PHC9564
RPMI 1640 Medium	Thermo Fisher Scientific, 11875093
B-27™ Supplement, minus insulin	Thermo Fisher Scientific, A1895601
XAV 939 (Reconstituted in DMSO to 10 mM and stored at -20 °C in 25 µl aliquots)	TOCRIS, 3748
KY 02111 (Reconstituted in DMSO to 10 mM and stored at -20 °C in 25 µl aliquots)	TOCRIS, 4731
B-27™ Supplement (50X), serum free	Thermo Fisher Scientific, 17504044
Retinoic Acid (Reconstituted in DMSO to 50 mM and stored at -80 °C in 5 µl aliquots)	Sigma-Aldrich, R2625-50MG
RPMI 1640 Medium, no glucose	Thermo Fisher Scientific, 11879020
CHIR 99021 (Reconstituted in DMSO to 10 mM and stored at -20 °C in 10 µl aliquots)	TOCRIS, 4423

IWR-1 (Reconstituted in DMSO to 10 mM and stored at -20 °C in 10 µl aliquots)	Sigma-Aldrich, I0161
Accutase® solution	Sigma-Aldrich, A6964
Advanced DMEM/F-12	Thermo Fisher Scientific, 12634010
Fibroblast Growth Medium 3	(PromoCell, C-23130)
Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein (Reconstituted in PBS (0.1 % BSA) to 10 µg/mL and stored at -80 °C in 50 µl aliquots)	bio-techne, 233-FB-010
SB431542 (Reconstituted in DMSO to 10 mM and stored at -80 °C in 50 µl aliquots)	TOCRIS, 1614

3.2.2 iPSC Culture

Induced pluripotent stem cells Kolfc2 (WTSIi018-B) were maintained on 6-well plates coated with Geltrex™ (1:100) (for details of reagents and suppliers see *Table 3.1*) according to the manufacturers protocol. Cells were cultured in mTeSR™ Plus media at 37 °C and 5 % CO₂ and passaged at 70-80 % confluency. Cells were passaged by removing the media in the well, washing once with PBS and adding TrypLE™ Express Enzyme (1X), phenol red for 3 minutes at 37 °C. The TrypLE Express was removed and the cells were gently washed off the surface of the well with 1 mL of warm mTeSR Plus medium containing 10 µM Rock Inhibitor (Y-27632). Cells were passaged according to a seeding density of 20,000 cells per cm² and cultured in 10 µM Rock Inhibitor (Y-27632) mTeSR Plus media for the first 24 hours before switching to mTeSR Plus. The Rho-kinase inhibitor (Rock Inhibitor), Y-27632 was added to prevent dissociation-induced apoptosis. It should be removed 24 hours after the passaging of the cells to maintain iPSC pluripotency.

3.2.3 iPSC-CM Differentiation

The method for differentiation of iPSC-CMs was broadly adapted from the protocol outlined in Smith et al., (2018) (*Figure 3.1*). iPSCs were seeded onto 6-well plates coated with Geltrex and cultured in 2 mL per well of mTeSR Plus. The medium was changed on the cells every 48 hours until the cells reached 60 % confluency. The medium was then changed for 24 hours

with StemPro™-34 SFM (1X) supplemented with 2 mM L-Glutamine, 1 ng/mL Recombinant Human BMP-4 Protein and 1:100 Geltrex. The cells were subsequently changed with StemPro medium supplemented with 10 ng/ml BMP-4, 8 ng/mL Activin A and 2 mM L-Glutamine and incubated for 48 hours (day 0). The medium was changed with RPMI 1640 Medium with B-27™ Supplement, minus insulin, 10 µM XAV 939 and 10 µM KY 02111 for 48 hours (day 2). The cells were subsequently changed with RPMI 1640 Medium with B-27™ Supplement (50X), 10 µM XAV 939 and 10 µM KY02111 for 48 hours (day 4). The medium on the cells was changed with RPMI 1640 Medium with B-27™ Supplement (50X) every other day. Atrial differentiation was achieved through the addition of 1.0 µM retinoic acid to the media on days 4 and 6. At 12 days after the initiation of differentiation, glucose starvation was performed to purify the population of cells for cardiomyocytes. This was achieved by changing the medium of the cells with RPMI 1640 Medium supplemented 1:50 with no glucose with B-27™ Supplement (50X) for 48 hours. Cells were dissociated at day 15 for incorporation into EHT models. The media was removed from the well(s), the well was washed once with 1 mL of PBS and the cells were incubated in 2 mL of TrypLE Select 10x for 30 minutes at 37 °C. The cells were then washed from the surface of the plate with pre-warmed DMEM-F12.

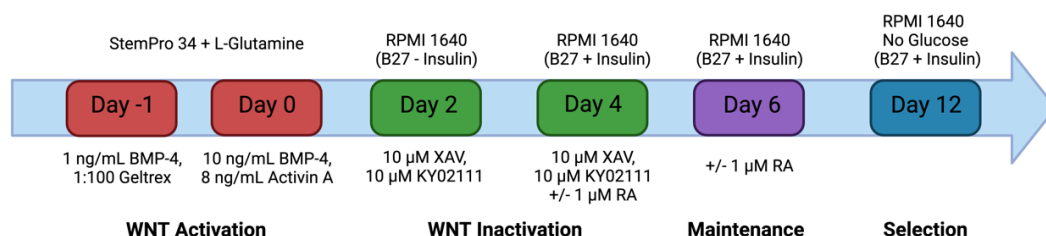


Figure 3.1: Differentiation of iPSC-CMs

Schematic representation of the iPSC-CM differentiation protocol. Mesodermal specification is achieved through the activation of the Wnt signalling pathway using the recombinant proteins: Bone morphogenic protein- 4 (BMP-4) and Activin A. Subsequent inactivation of the Wnt signalling pathway by small molecules XAV 939 and KY02111 enables the differentiation of cardiac progenitor cells into induced pluripotent stem cell derived cardiomyocytes. Differentiation can be driven towards atrial cardiomyocyte specification through the addition of retinoic acid on days 4 and 6 only. The medium on

the cells was changed every 48 hours after day 6 with RPMI 1640 with B27 + Insulin. Selection is achieved from days 12-14 by culturing the cells in RPMI 1640 No Glucose with B27 + insulin for 48 hours.

3.2.4 iPSC-CF Differentiation

The method for the differentiation of quiescent induced pluripotent stem cell derived cardiac fibroblasts was broadly adapted from the protocol outlined in Zhang et al., (2019) (*Figure 3.2*). Induced pluripotent stem cells (Kolfc2/WTSLi018-B) were seeded at 30,000 cells/cm² in a T25 flask pre-coated with 1:100 Geltrex for 2 hours at 37 °C, in 10 µM Rock Inhibitor (Y-27632) mTeSR Plus medium. The cells were cultured for 48 hours prior to the medium being changed with RPMI 1640 supplemented with B27 minus insulin and 6 µM CHIR-99021 for 48 hours. The medium was then changed with RPMI 1640 supplemented with B27 minus insulin for 24 hours. The cells were subsequently treated with a RPMI 1640 with B27 minus insulin and 5 µM IWR1 for 48 hours. The cells were dissociated by washing with 3 mL PBS and then treating with 5 mL Accutase enzyme for 3 minutes at 37°C. The cells were removed from the flask, diluted with 5 mL pre-warmed RPMI 1640 and spun down at 200 g for 3 minutes. The cells were resuspended in Advanced DMEM/F-12 supplemented with 5 µM CHIR-99021 and 2.0 µM Retinoic Acid and seeded at 20,000 cells/cm² in a Geltrex coated T25 flask. The cells were incubated in this medium for 3 days and then subsequently changed to Advanced DMEM/F-12 for a further 4 days. The cells were passaged and plated into a Geltrex coated T25 flask at a density of 20,000 cells/cm² in Fibroblast Growth Medium 3 supplemented with 10 ng/mL FGF2 and 10 µM of the TGF-β1 inhibitor SB431542. The cells were cultured in this media for 6 days, with media changes taking place every 48 hours. Following the 6 days, the cells can be assessed for purity using immunocytochemistry. It is recommended that the cells are expanded, passaged at 70-80 % confluency for 2 passages and cryopreserved for use in future experiments in serum free freezing medium. Cells were passaged by washing once with PBS, and then incubating in TrypLE™ Express for 3 minutes at 37 °C. Cells can be dislodged from the surface of the flask by gently tapping the sides of

the container. Following the detachment of the cells, a 2 x volume of DMEM/F12 was added to dilute the TrypLE. The TrypLE/cell mix was centrifuged at 300 g for 3 minutes. The cells were resuspended in Fibroblast Growth Medium 3 supplemented with 10 ng/mL FGF2 and 10 μ M SB431542 and seeded at a density of 10,000 cells/cm².

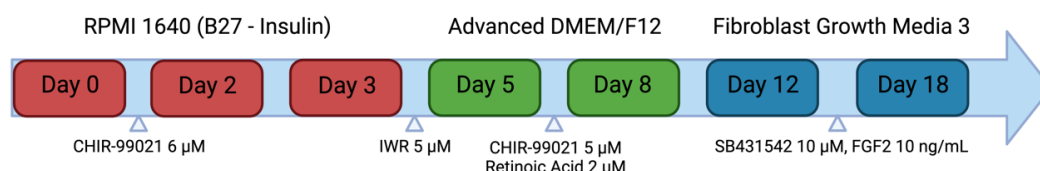


Figure 3.2: Differentiation of iPSC-CF

Schematic representation of the iPSC-CF differentiation protocol. Mesodermal specification is achieved through the activation of the Wnt signalling pathway using CHIR-99021. Subsequent inactivation of the Wnt signalling pathway by the small molecule IWR1 allows the generation of cardiac progenitor cells. Culture in CHIR-99021 and Retinoic Acid, drives the cells towards the derivation of cardiac fibroblasts. Cardiac fibroblasts are maintained in medium supplemented with FGF2 and the TGF- β 1 inhibitor SB431542 to prevent activation into myofibroblasts.

3.2.5 iPSC derived EHTs

3.2.5.1 Reagent Preparation

Table 3.2: List of Reagents, Suppliers, Preparation and Storage

Reagent/Media	Preparation and Storage
Silicon Rack (DiNAQOR, C0001)	Autoclave and keep sterile.
Teflon Spacer (DiNAQOR, C0002)	Autoclave and keep sterile.
UltraPure™ Agarose (Thermo Fisher Scientific, 16500100)	Dissolve agarose in sterile PBS to obtain a 2 % agarose solution. Autoclave and store at 4 °C.
10x DMEM, powder, high glucose (Thermo Fisher Scientific, 12100061)	Dissolve 1.34 g in 10 mL of sterile Tissue-Culture H ₂ O and sterilise by passing through a 0.22 μ m syringe filter (Merck Millex™-GP Sterile Syringe Filter, 10268401). Store at 4 °C for no longer than 1 week.
33 mg/mL Aprotinin (Sigma-Aldrich, A1153-100MG)	Dissolve 100 mg in 3.03 mL of sterile H ₂ O. Filter sterilise, divide into 50 μ L aliquots and store at -20 °C.
200 mg/mL Fib-Ap/Fibrinogen-Aprotinin Mix (Sigma-Aldrich, F8630-1G)	Gradually add 1 g of fibrinogen to 5 mL pre-warmed 0.9 % NaCl H ₂ O. <i>NOTE: Fibrinogen is difficult to dissolve in H₂O. Add small quantities of fibrinogen to the 0.9 % NaCl H₂O, warm in the incubator and</i>

	<i>place on the roller to ensure complete dissolution. Add 14.42 µl of aprotinin (33 mg/mL). Divide into 50 µL aliquots and store at -80 °C.</i>
100 U/mL Thrombin (Sigma-Aldrich, T4648-1KU)	Dissolve thrombin (1000 U) in 6 mL PBS and 4 mL H ₂ O. Divide into 50 µl aliquots and store at -80 °C.
5 mL of 2X DMEM (Make up fresh each time)	• 1 mL of 10x DMEM • 1 mL Gibco™ Horse Serum, heat inactivated, New Zealand origin (Thermo Fisher Scientific, 11540636) • 100 µl Penicillin-Streptomycin (Thermo Fisher Scientific, 11548876) • 2.9 mL sterile Tissue-Culture H₂O Sterile filter. Do not store.
Rock Inhibitor Y-27632 (Dihydrochloride) (Stem cell Technologies, 72304)	• Reconstitute in PBS to 10 mM, divide into 50 µl aliquots and store at -20 °C
NKM Medium	• 100 mL DMEM, high glucose (Thermo Fisher Scientific, 11965084) • 10 mL FBS (Sigma-Aldrich, F9665-50ML) • 1 mL Penicillin-Streptomycin (Thermo Fisher Scientific, 11548876) • 1 mL L-Glutamine (Thermo Fisher Scientific, 25030081) Store at 4 °C for no longer than 1 week.
EHT Medium	• 45 mL DMEM, high glucose (Thermo Fisher Scientific, 11965084) • 500 µl Penicillin-Streptomycin (Thermo Fisher Scientific, 11548876) • 50 µl Insulin (10 mg/mL) (Merck Millipore, 91077C-250MG) • 50 µl Aprotinin (33 mg/mL) (Sigma-Aldrich, A1153-100MG) Filter sterilise and store at 4 °C <i>Add 5 mL of horse serum (Thermo Fisher Scientific, 11540636) to 45 mL of above medium prior to use. The medium can be supplemented with 10 µM of the TGF-β1 inhibitor SB431542 (TOCRIS, 1614) to prevent cardiac fibroblast activation.</i>

Nunc™ Cell-Culture Treated 24 well plate (Thermo Fisher Scientific, 142475)	Other brands of 24 well plate have differing dimensions and will reduce success rate of EHT formation
TrypLE™ Select Enzyme (10X) (Thermo Fisher Scientific, A1217701)	Single cell dissociation is required for the generation of a homogenous EHT
0.5 ml TubeOne® Microcentrifuge Tubes, Natural (non-sterile) (StarLabs, S1605-0000)	Autoclave sterilised and pre-chilled.

3.2.5.2 Protocol for EHT construction

Here we describe a step-by-step protocol for the construction of 4 EHTs (1 strip) containing iPSC-CFs. The reagents required are listed in *Table 3.2*.

Preparation of reagents for EHT construction

1. Ensure there is premade sterile 2 % agarose in the fridge
2. Prepare and filter sterilise 2X DMEM mix, and leave on ice
3. Ensure there is premade NKM medium and chill on ice
4. Ensure there is premade EHT medium
5. Prewarm 40 mL of sterile DMEM-F12 in the water bath

Dissociation of iPSC-CMs

1. Remove media and wash 1-2 confluent wells of a 6 well plate containing iPSC-CMs with room temperature PBS once
NOTE: 1 x confluent well of iPSC-CMs in a 6 well plate should yield approximately 4×10^6 cells
2. Add 2 mL of TrypLE™ Select Enzyme (10X) per well of a 6 well plate and incubate for 30 minutes at 37 °C
3. Transfer the detached cells to a 50 mL centrifuge tube and wash off any attached cells with 1 mL per well of pre-warmed DMEM-F12
4. Add DMEM-F12 to the cell mix to give a total volume of 10 mL
NOTE: Centrifugation of iPSC-CMs takes place later, when both cell types have been combined
5. Perform a cell count

Dissociation of iPSC-CF (performed during Step 2 of iPSC-CMs dissociation)

1. Remove the media and wash a T25 flask (>80 % confluency) containing iPSC-CF with PBS
NOTE: A T25 flask at 80 % confluency should yield approximately 600,000 iPSC-CF
2. Add 3 mL TrypLE Express and incubate for 3 minutes at 37 °C
3. Gently tap the flask on all sides to dissociate cells from the surface of the flask
4. Transfer the 3 mL of TrypLE/cell mix to a 50 mL centrifuge tube
5. Rinse the flask with 7 mL of pre-warmed DMEM-F12 before combining it with the TrypLE containing cells
6. Perform a cell count

Combining cells and centrifugation

1. Combine required volumes of iPSC-CMs (4×10^6 cells) and iPSC-CF (6×10^5 cells) cell mixes in a centrifuge tube and make up to 30 mL with pre-warmed DMEM-F12
2. Spin down the tube containing both iPSC-CMs and iPSC-CF at 100 g for 10 minutes
3. Place thrombin, Fib-Ap, Rock Inhibitor and Geltrex on ice
4. Following centrifugation, gently place the cells on ice
NOTE: Be careful not to disturb the pellet

Making Agarose Moulds (performed during 10 minute centrifugation)

1. Microwave 2 % Agarose until completely molten
2. Pipette 1.6 mL of molten agarose per well in a column of a Nunc 24 well plate
3. Insert a Teflon spacer into a column of the Nunc 24 well plate containing molten agarose to create agarose moulds for a silicon rack of 4 EHTs
NOTE: Wells of a 24 well plate can differ in dimensions. The spacers and silicon racks are optimised for use in Nunc™ Cell-Culture Treated 24 well plates
4. Remove the Teflon spacers from the now solidified agarose moulds after 15 minutes
NOTE: Agarose moulds can deteriorate and crack over time. Cast EHTs in the agarose moulds <10 minutes after removal of the Teflon spacers
5. Place the silicon racks into the moulds, ensuring central alignment
NOTE: Misaligned silicon racks increase the risk of snagging during removal from the moulds

Preparing the EHT Mix (See Table 3.3)

1. Aliquot 3 µl of the thrombin into 4 x 0.5 mL microcentrifuge tubes and chill on ice (4 tubes for 4 EHTs)
NOTE: Briefly spin down the tubes containing the thrombin
2. Add 368.55 µl of NKM medium to a prechilled 1.5 mL microcentrifuge tube
3. Prewarm the Fib-Ap mix in your hands (to enable accurate pipetting) and then add 11.25 µl to the NKM medium
NOTE: Complete dissolution of the Fib-Ap mix is paramount to successful generation of EHTs.
4. Add 24.75 µl of 2X DMEM
5. Add 0.45 µl of Rock Inhibitor
6. Add 45 µl of geltrex
7. Resuspend the cell pellet containing both the iPSC-CMs and iPSC-CF in the EHT mix
NOTE: Everything should be kept on ice at this stage.

Casting EHTs

1. Add 100 µl of the EHT cell mix to a 3 µl aliquot of thrombin and then quickly pipette the mix in between the 2 silicon posts resting inside the agarose mould
NOTE: Minimise risk of air bubble formation by only pipetting down to the first stop of the pipette
2. Repeat step 1 for each EHT
NOTE: Be careful not to knock or disturb the 24 well plate holding the solidifying EHTs
3. Gently triturate the EHT cell mix intermittently (every 4 EHTs) to ensure homogeneity
4. Gently place the lid on the 24 well plate and place in a 37 °C incubator for 2 hours

Removal and Culture of EHTs

1. Warm NKM medium in a 37 °C water bath
2. Add 1.5 mL of EHT medium to each well of a column of wells in a Nunc 24 well plate and place in the 37 °C incubator
3. After 2 hours, gently pipette 350 µl of prewarmed NKM medium on top of the agarose mould in each well
NOTE: Pipetting on top of the agarose mould prevents disruption to the EHT
4. Place in the incubator for a further 10 minutes
5. Move the plate from side to side to encourage dislodging of the EHTs
6. Carefully but firmly remove the EHT strips from the agarose moulds and place into the wells containing the prewarmed EHT medium
NOTE: Removal of the strips should be performed in a single action as prolonged and gentle removal of the EHTs from the agarose moulds can increase the risk of snagging and malformation
7. Maintain the EHTs by transferring the silicon racks into wells containing fresh EHT medium every 48 hours.

Table 3.3: EHT Mix Composition

Component	1X	4.5X (Mastermix for 4 EHTs)
1: NKM Medium	81.9 µl	368.55 µl
2: 200 mg/mL Fib-Ap Mix	2.5 µl	11.25 µl
3: 2X DMEM (made up fresh)	5.5 µl	24.75 µl
4: Rock inhibitor (10 mM)	0.1 µl	0.45 µl
5: Geltrex	10 µl	45 µl

3.2.6 RT-qPCR

iPSC-CFs were cultured in T25 flasks for 72 hours in Fibroblast Growth Media 3 supplemented with 10 ng/ml FGF2 + 10 µM SB431542 or 10 ng/ml FGF2 + 10 ng/mL TGF-β1 (R&D Systems, 240-B-002/CF). RNA extraction was performed using the Direct-zol RNA Miniprep Kit (Zymo Research, R2050) according to the manufacturer's protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) according to the manufacturer's protocol. RT-qPCR was performed using TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557) following the manufacturer's protocol and on a QuantStudio™ 5 Real-Time PCR System, 384-well (Thermo Fisher Scientific, A28140). RT-qPCR was performed using TaqMan™ Gene Expression Assay (FAM) (Thermo Fisher Scientific, 4331182) for ACTA2 (Hs00426835_g1) and COL1A1 (Hs00164004_m1). All reactions were multiplexed and

normalised to the housekeeping gene GAPDH using Human GAPD (GAPDH) Endogenous Control (VICTM/MGB probe, primer limited) (Thermo Fisher Scientific, 4326317E). 10 ng of cDNA was used per reaction. Transcript levels were measured from 3 samples of iPSC-CFs, with each sample being obtained from cells at different passages. Cycle threshold values were obtained from 3 technical replicates of each sample. Relative gene expression was calculated using the comparative cycle threshold method (Schmittgen et al., 2008).

3.2.7 Immunofluorescence

3.2.7.1 iPSC-CFs

iPSC-CFs were split into wells of a Geltrex coated 24 well plate (10,000/well) and cultured for 72 hours in Fibroblast Growth Media 3 supplemented with 10 ng/ml FGF2 + 10 μ M SB431542 or 10 ng/ml FGF2 + 10 ng/mL TGF- β 1. Cells were fixed by for 14 minutes at room temperature using 4 % paraformaldehyde Solution (Thermo Fisher Scientific, J19943.K2). Fixed cells were blocked for 1 hour in blocking buffer consisting of PBS, Fetal bovine serum (5 %), Bovine Serum Albumin (1 %) (Merck Millipore, A9418-10G) and TritonTM X-100 (0.5 %) (Merck Millipore, X100-100ML). Primary antibodies were added at dilutions listed in *Table 3.4* in blocking buffer for 2 hours at room temperature. This was followed by 3 washes in blocking buffer and the addition of secondary antibodies (*Table 3.4*) for 1 hour in the dark at room temperature. DAPI (Thermo Fisher Scientific, D1306) was added with the secondaries at 0.1 μ g/mL to visualise nuclei. Samples were washed 3 times in blocking buffer and then once with PBS. Samples were imaged using a EVOS M5000 Imaging System (Thermo Fisher Scientific, AMF5000).

3.2.7.2 Engineered Heart Tissues

EHTs constructed with and without the addition of iPSC-CFs were removed from posts after 15 days in culture (30 days post initiation of differentiation), washed 3 times in PBS and

fixed overnight at 4 °C in 4 % PFA. A scalpel was used to longitudinally section EHTs. Sectioned pieces of EHTs were blocked overnight in blocking buffer. Primary antibodies were added in blocking buffer, at the dilutions listed in *Table 3.4*, overnight at 4 °C. Samples were washed 3 times in blocking buffer before the addition of secondary antibodies for 4 hours in the dark at room temperature. DAPI was added with the secondaries at 0.1 µg/mL to visualise nuclei. Tissue sections were placed onto coverslips (VWR, 631-0170) and mounted onto cover slides using Hydromount Histology Mounting Media (Scientific Laboratory Supplies, NAT1324). Images were acquired using a Zeiss LSM780 Confocal laser scanning microscope equipped with a C-Apochromat 63x/1.20 W objective.

Table 3.4: Primary and Secondary Antibodies

Target	Company, Catalog	Host	Concentration
Vimentin (Primary)	Abcam, ab45939	Rabbit	1:500
α-Smooth Muscle Actin (Primary)	Merck Millipore, A5228	Rabbit	1:500
Collagen 1 (Primary)	Abcam, ab34710	Rabbit	1:500
Titin (T12) (Primary)	Fürst et al., 1988	Mouse	2 µg/mL
Alexa Fluor™ 488 anti-Mouse IgG (Secondary)	Thermo Fisher Scientific, A28175	Goat	1:500
Alexa Fluor™ 546 anti-Rabbit IgG	Thermo Fisher Scientific, A-11035	Goat	1:500
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Nuclear Stain)	Thermo Fisher Scientific, D1306	N/A	0.1 µg/mL

3.2.8 Analysis of Contractile Function

Engineered heart tissues constructed with and without iPSC-CFs were cultured for 15 days in EHT media (30 days after initiation of differentiation) prior to recording and analysis. 10 second videos of each EHT was recorded at 1080p/30 fps using an inverted camera with a macroscopic lens (3 EHTs with iPSC-CF, 3 EHTs without iPSC-CF). Videos were converted into tif files and analysed using the ImageJ Plugin TrackMate for displacement over time. All

EHTs with contractile function were included in the analysis. Contractile function was defined as at least two macroscopically visible contractions within 15 seconds. All EHTs constructed with iPSC-CFs demonstrated contractile function. Only 3 of the 9 EHTs constructed (1 of 3 batches, 3 EHTs per batch) without the addition of iPSC-CFs showed contractile function. The analysis of contractile function was performed blinded to the presence or absence of iPSC-CF. Outputs were semi-automated and included contraction duration, time to peak, relaxation time, total contraction amplitude and beat rate. For converting displacement into force, elastic bending of the pillars was assumed with a measured Young's modulus of 3.0 MPa, yielding a conversion factor of 0.26 mN/mm.

3.3 Results

3.3.1 Generation of Quiescent iPSC-CF

iPSCs were differentiated into cardiac fibroblasts using the protocol outlined in the materials and methods. Following differentiation, the activation status and plasticity of the cells was assessed using immunofluorescence. The cells were cultured in the presence and absence of 10 ng/mL TGF- β 1 for 72 hours. The cells were then fixed and stained for vimentin, α SMA and collagen 1 with the results shown in *Figure 3.3A*. Vimentin is a cardiac fibroblast marker irrespective of activation status whilst α SMA is predominantly expressed in activated or myofibroblasts. Collagen 1 can show expression in quiescent cardiac fibroblasts but is broadly associated with activated cardiac fibroblasts. The iPSC-CFs cultured in the absence of TGF- β 1 were positive for vimentin but negative for α SMA, indicating a quiescent cardiac fibroblast phenotype. The cells cultured without the TGF- β 1 inhibitor, SB431542, and in the presence of 10 ng/mL TGF- β 1 were positive for vimentin and α SMA and showed greater expression of collagen. The gene expression of *ACTA2* (the transcript related to α SMA) and *COL1A1* (a transcript related to collagen) was assessed using RT-qPCR as markers for cardiac fibroblast activation. Expression analysis was performed on 3 biological samples, with 3 technical repeats performed for each sample. No data was excluded from the analysis. The results are shown in *Figure 3.3B*. The iPSC-CFs showed a 3-fold increase in the mean expression of *COL1A1* following TGF- β 1 treatment (-TGF- β 1: M = 1, SD = 0.21, +TGF- β 1 M = 2.9, SD = 0.47). The mean expression of *ACTA2* in the iPSC-CFs increased approximately 5-fold following TGF- β 1 treatment (TGF- β 1: M = 1, SD = 0.13, +TGF- β 1 M = 4.9, SD = 1.59).

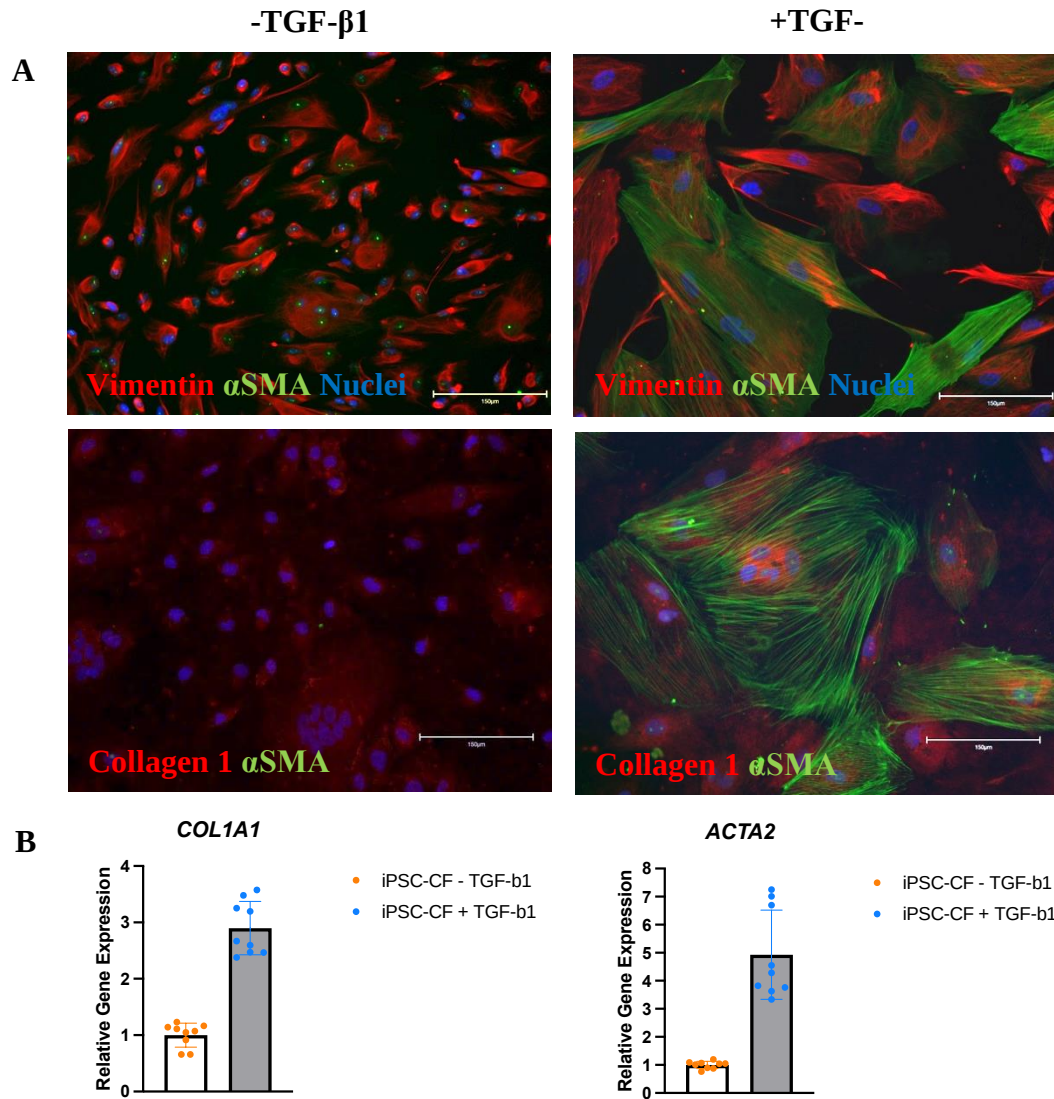


Figure 3.3: Activation State of iPSC-CFs

iPSC-CFs were cultured in the standard media (FGM3 + 10 ng/ml FGF2 + 10 μ M SB431542, ‘-TGF- β 1’) and with 10 ng/mL TGF- β 1 instead of the SB431542 (‘+TGF- β 1’) for 72 hours to assess activation status. **A:** The cells were fixed and stained for vimentin (top red), Collagen (bottom red) α SMA (green) and DAPI (blue, indicating nuclei). Scale bar = 150 microns. **B:** RNA was extracted, and RT-qPCR was performed on the cDNA for ACTA2 and COL1A1. N = 3 (3 biological samples with 3 technical replicates per sample are plotted).

3.3.2 Macroscopic EHT Structure

A workflow delineating the generation of EHTs from iPSCs is displayed in *Figure 3.4*. EHTs containing iPSC-CMs and iPSC-CFs started twitching approximately 48 hours after construction, with maximal beating capacity being reached after approximately 14 days in culture. Those generated in the absence of iPSC-CFs started twitching approximately 96 hours after construction and showed diminished contractile function throughout the duration of culture. EHTs constructed with and without iPSC-CF demonstrated marked differences in

shape and structure after 72 hours in culture as shown in *Figure 3.5*. EHTs constructed with iPSC-CFs showed tightening around the silicon posts whilst iPSC-CM only EHTs remained more block like throughout time in culture.

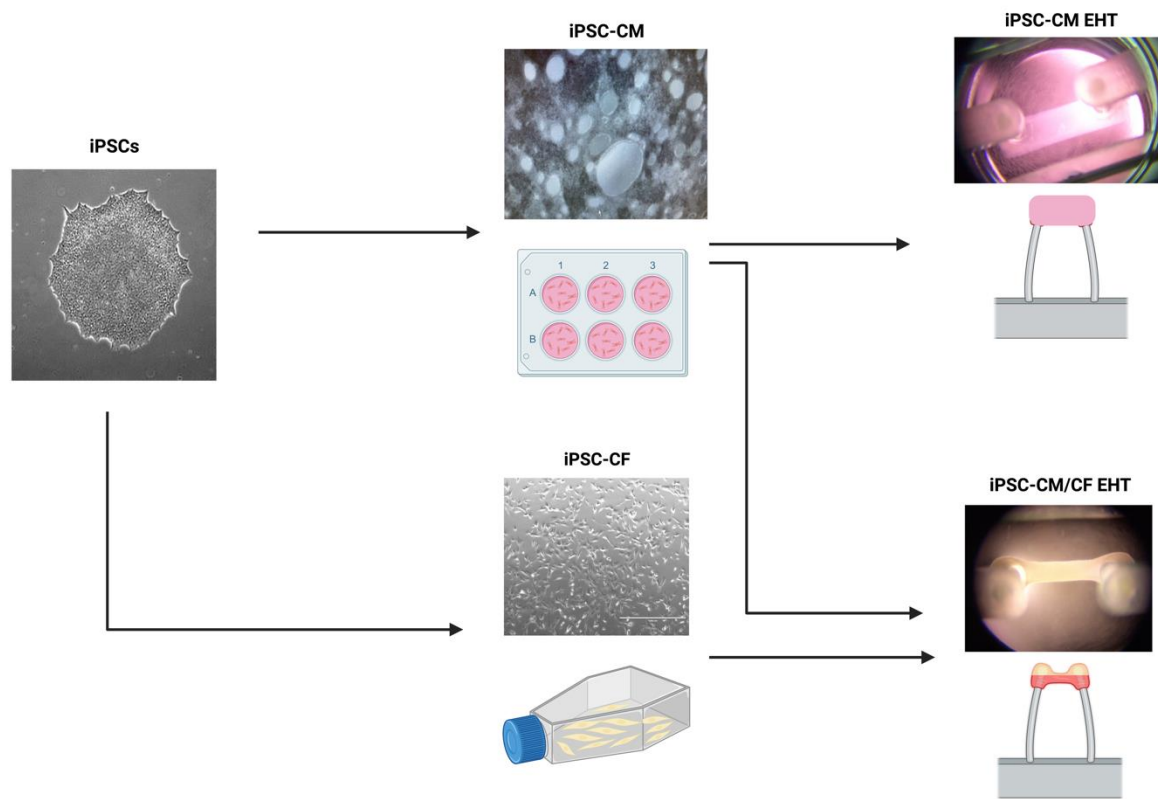


Figure 3.4: Generation of Engineered Heart Tissues

An outline of the workflow to generate of EHTs with and without the addition of iPSC-CFs. The EHT shown on the top right was generated without the addition of iPSC-CFs. The EHT shown on the bottom right was constructed with the addition of iPSC-CFs.

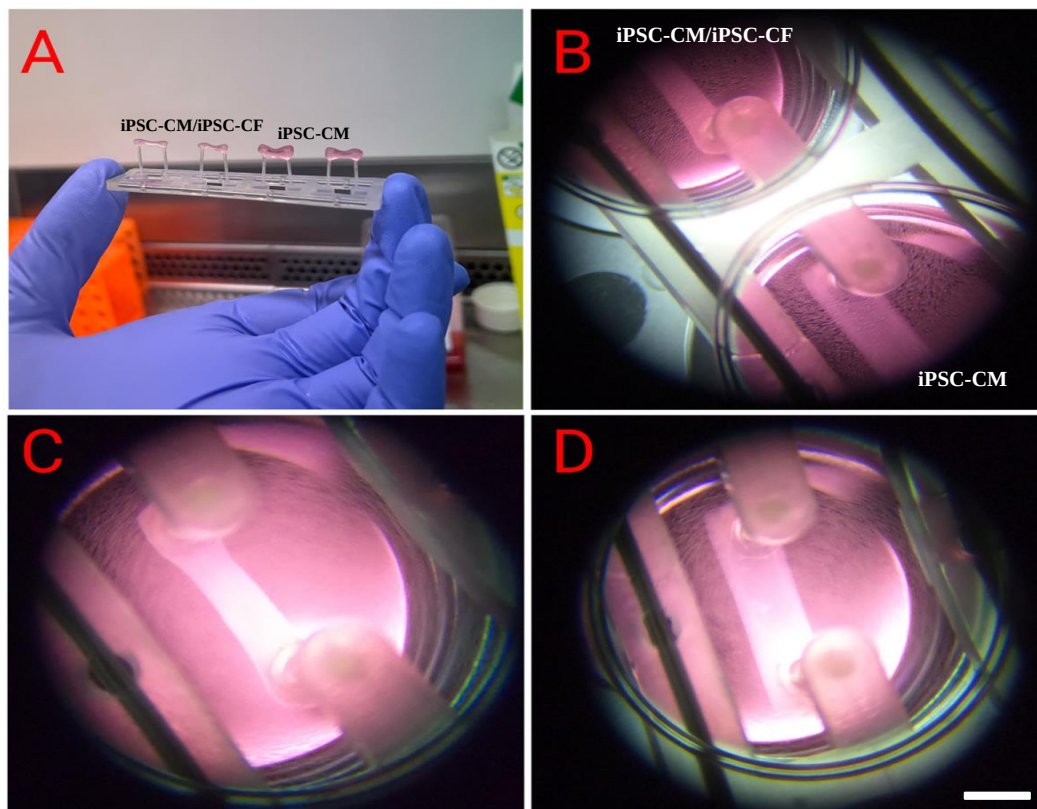


Figure 3.5: Morphology of EHTs 72 Hours After Construction

EHTs were constructed with and without the addition of iPSC-CFs according to the protocol outlined above. **A** and **B** show side by side comparisons. **C** - Close-up of an EHT with iPSC-CFs and iPSC-CMs demonstrated compaction after 72 hours, which was not observed in EHTs generated from iPSC-CMs alone (**D**). Scale bar = 4 mm

3.3.3 Contractile Analysis of EHTs

At day 30 after the initiation of differentiation (15 days in EHT form), 1080p/30 fps videos of EHTs with and without iPSC-CFs were analysed using the Open-source ImageJ plugin TrackMate (*Figure 3.6*). All EHTs which demonstrated contractility were included in the analysis. EHTs with and without iPSC-CFs demonstrated similar contraction durations and relaxation times of approximately 400 and 240 ms respectively. The mean time to peak of the EHTs containing iPSC-CFs was similar to that of their iPSC-CM only counterparts at approximately 170 ms. The average contraction amplitude of the EHTs containing iPSC-CF was approximately 30 % greater than that of the EHTs consisting of iPSC-CMs only. EHTs constructed with iPSC-CF demonstrated a marked decrease in beat rate.

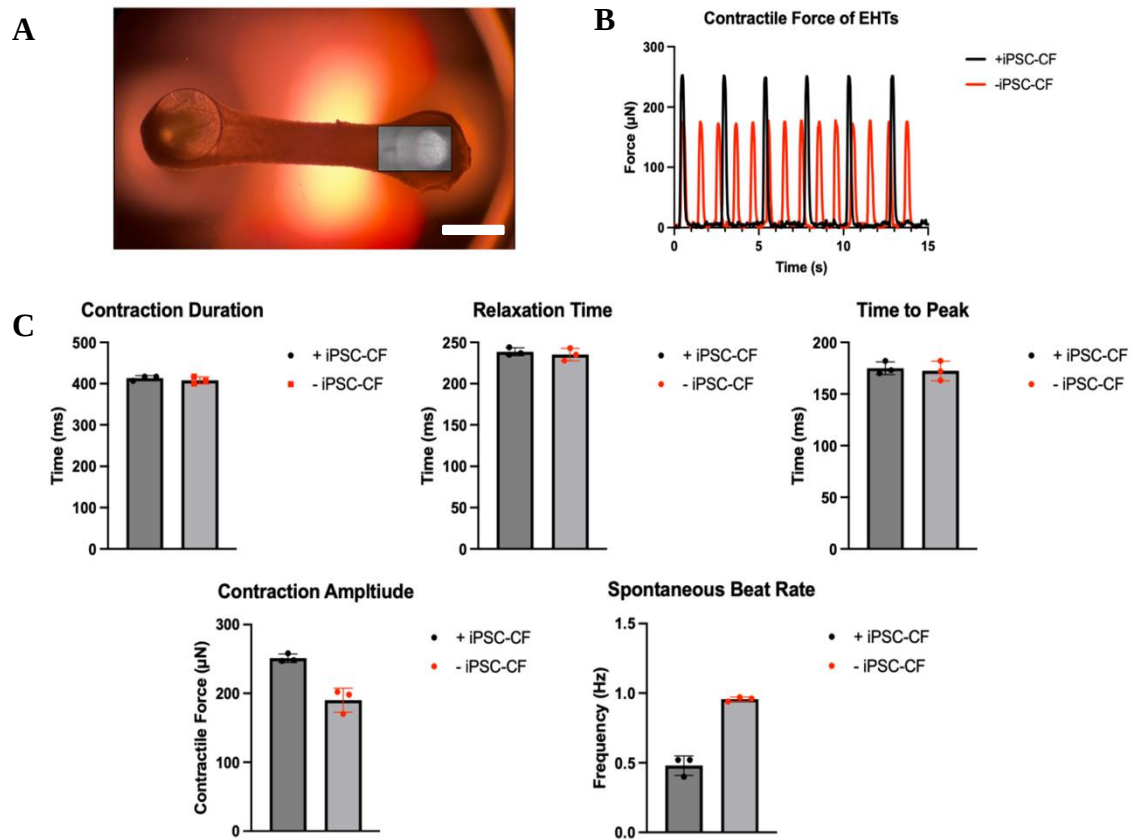


Figure 3.6: Contractile Analysis of EHTs

EHTs constructed with and without the addition of iPSC-CFs were analysed to assess contractile activity. Frames of the videos of the EHTs were analysed using the ImageJ Plugin TrackMate for displacement over time, the box represents the analysis window where movement of the pillar was tracked (A). Contractile profiles were generated (a representative profile of an EHT with and without iPSC-CFs is shown) (B) and used to ascertain Contraction Duration, Relaxation Time, Time to Peak, Contraction Amplitude and Spontaneous Beat Rate (C). N = 3 EHTs. Scale bar = 4 mm.

3.3.4 Composition of iPSC derived Engineered Heart Tissue

EHTs consisting of iPSC-CMs and iPSC-CMs/iPSC-CFs were cultured for 15 days after construction (30 days after initiation of iPSC-CM differentiation). The tissue was fixed and sectioned using a scalpel and then stained for DAPI, titin and vimentin to assess composition and alignment (Figure 3.7). Vimentin is a key marker for cardiac fibroblasts whilst titin is an integral component of the sarcomeres of cardiomyocytes. The iPSC-CMs demonstrated longitudinal alignment in EHTs constructed with and without iPSC-CF (Figure 3.7). Cells stained positive for vimentin in the EHTs constructed with iPSC-CFs but were absent in the iPSC-CMs only tissues.

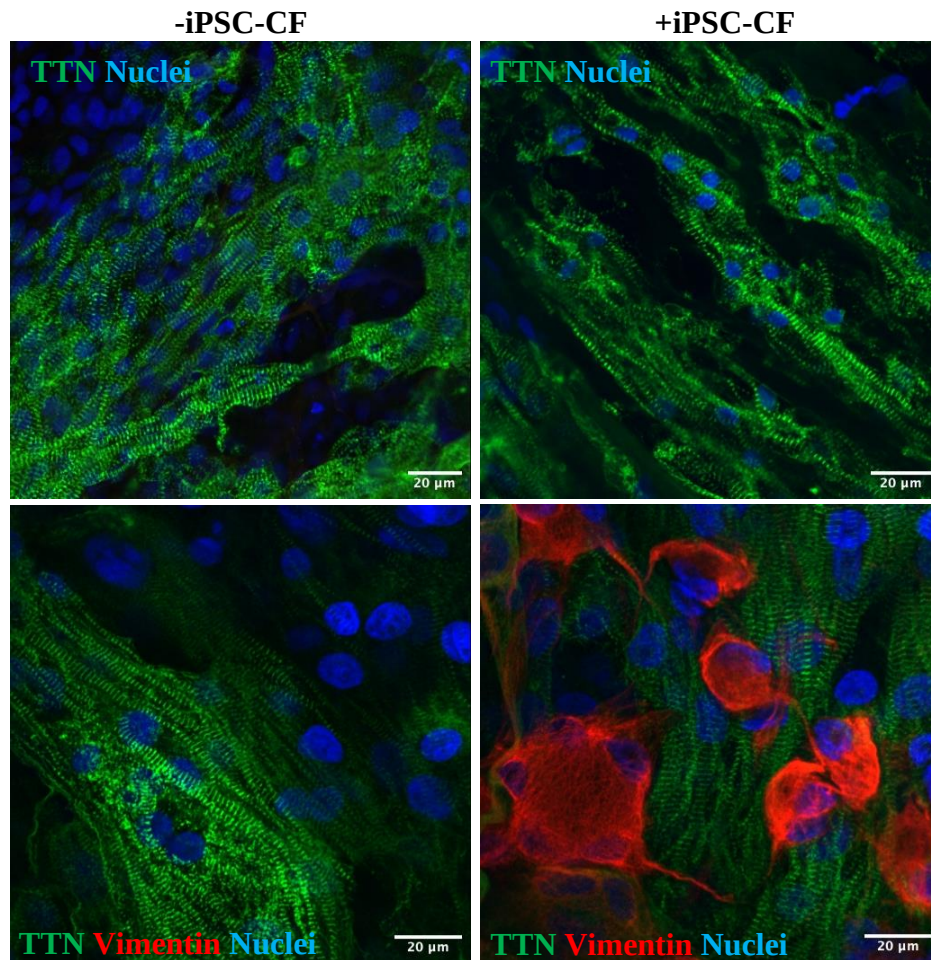


Figure 3.7: Alignment and Composition of Engineered Heart Tissues

EHTs were constructed with and without iPSC-CFs. Tissues were fixed, sectioned and stained for Titin (TTN) and DAPI (**top**) and Titin (TTN), Vimentin and DAPI (**bottom**). Scale bar = 20 microns.

3.4 Discussion

In this study we describe efficient and reproducible methods for the generation of iPSC-CMs and iPSC-CFs. Furthermore, we detail the subsequent integration of these cells into co-culture EHTs with improved contractile function. This approach has the potential to explore the complex interplay between cardiomyocytes and cardiac fibroblasts in the pathophysiology of heart disease. The iPSC-CFs generated were quiescent under the culture conditions outlined in the protocol, with plasticity demonstrated through the addition of the pro-fibrotic cytokine TGF- β 1. EHTs constructed with the addition of iPSC-CFs exhibited anisotropic alignment, decreased beat rate, increased tissue compaction and enhanced contractile force. The importance of non-myocyte cell types in 3D cardiac constructs is well established and has been widely discussed in previous publications (Kofron and Mende, 2017). EHTs consisting of both iPSC-CMs and iPSC-CFs demonstrated an increase in tissue compaction following just 72 hours in culture and a decrease in beat rate after 2 weeks in culture. Tissue compaction plays an important role in determining contractile capability and likely occurred in the EHTs constructed with iPSC-CFs due to the regulatory function that cardiac fibroblasts have on the extracellular matrix (Rivera-Arbeláez et al., 2022) (Thavandiran et al., 2013). A decrease in beat rate was observed from EHTs that were constructed with iPSC-CF. This is consistent with previous studies that have demonstrated lower beating rate frequencies in iPSC-CM/fibroblast co-culture models and is likely due to increased CM maturity and decreased funny current expression (Ronaldson-Bouchard et al., 2018).

EHTs constructed in this study from metabolically purified populations of iPSC-CMs, without the addition of iPSC-CFs, demonstrated diminished or no contractile function. Functional and contractile EHTs consisting solely of iPSC-CMs were generated during the course of this study but with a significantly reduced rate of success. The presence of non-myocyte cell types in the tissue population has previously shown to be beneficial to the

successful generation and improved contractility of 3D cardiac models (Naito et al., 2006) (Ravenscroft et al., 2016). Some studies have robustly demonstrated the successful generation of EHTs using CM populations with a purity of 92-100 % (Mannhardt et al., 2016) (Mannhardt et al., 2020). Differences in stem cell line, iPSC-CM differentiation, handling, and maturation may help explain contrasting success rates in iPSC-CM only EHT construction. The further development of 3D cardiac models consisting of defined populations of cardiac myocytes and stromal cells, e.g., fibroblasts, will help to reduce batch to batch variability and contribute to the development of more reliable *in vitro* modelling systems.

The relative ease by which iPSCs can be genetically engineered has allowed researchers to efficiently interrogate the effects pathogenic variants have in the cardiac myocyte. The development of isogenic EHTs consisting of iPSC derived cardiomyocytes and cardiac fibroblasts provides a more physiological model in which mutations can be explored and presents increased opportunity for exploring variants with pathological effect in both cell types (Zou et al., 2022). Furthermore, such models represent an exciting prospect for the future incorporation into pre-clinical drug screening and may eventually help relieve the current reliance on *in vivo* models.

Differences in the intrinsic electrophysiological properties of mouse cardiac myocytes have hampered efforts to model complex cardiovascular diseases. Furthering our understanding of currently enigmatic aspects of human cardiac disease, such as the dynamic interplay present between cardiomyocytes and fibroblasts in cardiac fibrosis, likely requires physiological and ultimately human models, such as the EHTs applied in this study. Improvements to human *in vitro* modelling systems are required to understand the complexities of cardiac pathology and will likely aid in the reduction and/or replacement of animal models in cardiac research.

CHAPTER 4: Atrial fibrillation-associated titin truncating variant TTN c.59926+1 G>A activates atrial profibrotic signalling and augments sensitivity to cholinergic stimulation

The following will be submitted as a research article. Contractility analysis on videos of EHTs was performed by Mr Jonas Euchner (University of Birmingham). Computational modelling of the TTNtv was performed by Dr Albert Dasí and Ms Leto Riebel. Editing of the TTNtv into iPSCs was performed by Dr Naeromit Sontayananon (*Figure 4.1, 4.S1, 4.S2, 4.S3 and 4.S5*). All authors have helped with revisions of this manuscript.

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4.1 Introduction

Atrial Fibrillation (AF) remains the most commonly sustained cardiac arrhythmia and is a significant risk determinant for the development of stroke, heart failure and sudden cardiac death (Wolf et al., 1991) (Maisel and Stevenson, 2003) (Waldmann et al., 2020). It is characterised by incidents of disordered and erratic signal propagation in the atria. Obesity, age, heart disease and hypertension are associated with structural and electrophysiological changes to the atria and the consequent development and maintenance of AF (Lau et al., 2017) (Fabritz et al., 2016). In addition, AF has a strong hereditary component, driven by both common and rare genetic variants (Ellinor et al., 2010) (Yoneda et al., 2021) (Roselli et al., 2018). Rare genetic variants associated with early-onset AF are clustered in genes coding for proteins of the cardiomyocyte contractile apparatus (Andersen et al., 2021). Recently, a clear link has emerged between new-onset AF and genetic variants that cause a truncation in the giant sarcomeric protein titin (*TTN*tv) (Ahlberg et al., 2018). Furthermore, patients with *TTN*tv associated dilated cardiomyopathy (DCM) have an increased burden for arrhythmia, with AF often being one of the first clinical manifestations of the disease (Corden et al., 2019) (Tayal et al., 2017) (Vissing et al. 2021). The mechanisms linking *TTN*tv with AF are poorly understood. Studies in induced pluripotent stem cell derived ventricular cardiomyocytes (iPSC-CMs) have demonstrated cardiomyopathy-associated changes including sarcomere disorganisation and contractile dysfunction (Huang et al., 2023) (Hinson et al., 2015). Here we created atrial iPSC-CMs carrying a *TTN*tv associated with DCM and AF *TTN* c.59926+1 G>A (rs553526525).

A rare titin truncating variant *TTN* c.59926+1 G>A (rs553526525) described in Hoorntje et al., (2018) is linked to the autosomal dominant development of DCM and early onset familial AF. The variant, which is located in a splice site in the intron traversing exons 303 and 304 of the A-band, putatively causes a truncation of titin by disrupting alternative mRNA splicing.

Retrospective clinical analysis revealed that half of all carriers developed early-onset AF. Furthermore, 30 % of carriers that did not present with DCM developed atrial tachyarrhythmia and fibrillation, making the variant a significant risk factor independent of gross pathoanatomical change. The mechanism(s) conferring the increased burden of atrial arrhythmia in patients with TTNtv remains unresolved. A better understanding should provide important information relating to prevention and treatment of AF in patients with TTNtv.

To explore the effect the c.59926+1 G>A TTNtv has on atrial cardiomyocyte function and structure we generated a novel TTNtv carrying iPSC line using CRISPR/Cas9 engineering. In atrial iPSC-CMs, the TTNtv impaired sarcomere formation and increased expression of the pro-fibrotic stress markers. TTNtv carrying atrial EHTs demonstrated a significant increase in extracellular matrix protein secretion. The TTNtv caused increased spontaneous action potential frequencies and activity of the acetylcholine-sensitive K^+ current (I_{KACh}). The data derived suggests that TTNtv confer an increased risk to fibrotic structural remodelling of the atria, which is a common feature of AF. *In-silico* simulations, conducted in a detailed 3D human atrial model, indicate that the I_{KACh} up-regulation observed for the TTNtv creates a heterogeneous repolarisation substrate that is sufficient to propitiate AF, even in the absence of structural remodelling or ectopic triggers.

4.2 Materials and Methods

4.2.1 CRISPR/Cas9-mediated generation of *TTN*tv iPSCs

The KOLF2-C1 (WTSli018-B-1) iPSC line which was used for all experiments in this study was provided by the Wellcome Sanger Institute. The reconstituted ribonucleoprotein complex of 1 µl of 1 µg/µl guide RNA containing *TTN* c.59926+1 targeting crRNA (5'-AGAGAAAGCAACUUACGGAG -3'; IDT) and 0.6 µl of 20 µM Alt-R® S.p. HiFi Cas9 Nuclease (IDT) was electroporated into iPSCs in the presence of a HDR donor carrying *TTN* c.59926+1 G>A (*TTN*tv) and *TTN* c.59910 G>A (silent *Hind*III site) (Thermo Fisher Scientific). Isolated colonies of the electroporated cells were established and genotyped by genomic PCR using primers encompassing both the modification sites (F: 5'-AACCTGGGCCACCTAGAGAT-3', R: 5'-GGAGACCAAGACCCACAATG-3', 637-bp product). Specific *Hind*III digestion pattern on the PCR amplicons (280 and 357 bp) allowed identification of integration events of the HDR donor, which was subsequently verified by Sanger DNA sequencing. Successful colonies were validated as pluripotent and normal iPSCs by FACS analysis for expression of stem cell markers including OCT4, SOX2, NANOG and SSEA4 using Human and Mouse Pluripotent Stem Cell Analysis Kit (BD Biosciences, *Figure 4.S1*) and karyotyping by chromosomal counting at metaphase (*Figure 4.S2*) performed by the Chromosome Dynamics Core Facility at the Wellcome Centre for Human Genetics, UK.

4.2.2 iPSC Culture and Differentiation into Atrial Cardiomyocytes

iPSCs were cultured and differentiated into atrial iPSC-CMs as previously described in (Cumberland et al., 2023). Briefly, iPSCs were cultured in mTeSR Plus medium under humidified conditions at 37 °C and 5 % CO₂. Cells were passaged at 80 % confluency onto Geltrex®-coated 6-well dishes, in mTeSR Plus medium supplemented with 10 µM of the

ROCK inhibitor Y-27632. iPSCs were differentiated into cardiac progenitor cells through activation of the WNT signalling pathway using 10 ng/mL BMP-4 and 8 ng/mL Activin A. WNT inhibition and cardiomyocyte derivation was achieved through the addition of 10 μ M XAV 939 and 10 μ M KY 02111. Atrial specification was achieved through the addition of 1.0 μ M retinoic acid on days 4 and 6 of differentiation. Cardiomyocyte metabolic selection was carried out through glucose starvation for 48 hours, 12 days after the initiation of differentiation. The iPSC-CMs were passaged by washing once with PBS and incubating in TrypLE™ Select Enzyme (10X), no phenol red (Thermo Fisher Scientific, A1217702) for 30 minutes. Cells were washed off with pre-warmed DMEM/F-12 (Thermo Fisher Scientific, 11320033) and centrifuged for 10 minutes at 100 $\times g$. Cells were counted using a Neubauer and seeded at 10,000 cells/cm².

4.2.3 Atrial/Ventricular iPSC-CM Analysis

Analysis on the successful generation of atrial iPSC-CMs was performed by assessing the presence of structural, genetic and electrophysiological markers. Action potentials were recorded from a single batch of atrial (1.0 μ M retinoic acid on days 4 and 6 of differentiation) and ventricular iPSC-CMs. Stimulated action potentials recorded from atrial iPSC-CMs showed a slower upstroke, a shortened APD and more atrial-like action potential morphology. Atrial iPSC-CMs showed upregulation of the atrial-specific ion channel subunit gene *KCNJ3*. Structural expression of the ventricular/atrial specific isoform of the myosin light chain (MLC) was analysed by immunofluorescence. Atrial and ventricular iPSC-CMs were stained for both MLC-2a and MLC-2v. Images were acquired at random and the number of cells positive for each or both of the stains were counted in each image. The proportion of cells positive for each marker are shown in *Figure 4.S4*.

4.2.4 Engineered Heart Tissues

4.2.4.1 *Generation and Culture of iPSC-derived Engineered Heart Tissues*

Atrial engineered heart tissues (EHTs) were generated using iPSC-derived cardiac fibroblasts (iPSC-CFs) and iPSC-CMs as detailed in Cumberland et al., (2023). In brief, iPSC-CFs were differentiated from KOLF2-C1 iPSCs using a protocol adapted from Zhang et al., (2020) and retained in a quiescent state through the addition of 10 μ M SB431542. Day 15 atrial iPSC-CMs were combined with iPSC-CFs (1×10^6 iPSC-CMs and 1.5×10^5 iPSC-CFs) to form a co-culture EHT. The EHTs were cultured until 30 days after the initiation of differentiation in media containing 10 μ M of the TGF- β 1 inhibitor SB431542 to prevent chronic activation (Landry et al., 2019). The TGF- β 1 inhibitor was removed on day 30 to assess activation over a defined period. Tissue/supernatant was harvested on day 35 (5 days without the TGF- β 1 inhibitor).

4.2.4.2 *Contractility Analysis of Engineered Heart Tissues*

A combination of semi-automated object tracking with automated parameter extraction was used for the analysis of EHT contractile kinetics. Code available from (<https://gitfront.io/r/TrackerEHT/wF8g1EfA7LfD/EHT-pillarTracker/>). EHTs were cultured for 15 days in EHT media. Each EHT was analysed for >10 seconds using an inverted microscope with a 1x custom-built air spaced objective. Contractions were recorded at 60 FPS. The analysis is conducted using a two-tier approach. First, an ImageJ script iteratively processes each file, performing object tracking and trace extraction. This script involves user intervention to identify the objects of interest, which is then tracked through successive image slices using template matching. Parameters such as maximal frame-to-frame movement and object size are configurable by the user. The extracted tracks are then analysed using R. The script iteratively processes each file, extracting movement data and calculating relevant parameters, including contraction duration, time to peak, relaxation time, contraction

amplitude, contraction force, and peak to peak time. Traces are baseline corrected to compensate for any drift in acquisition. Peaks are fitted on a smoothed trace, detecting peak amplitude, peak position, peak start, and peak end using the “pracma” package for peak fitting. To convert the measured displacement into contraction force, a conversion factor of 0.26 mN/mm was determined (Cumberland et al., 2023).

4.2.5 RT-qPCR

RNA was harvested from 2D iPSC-CM samples at 30 days after the initiation of differentiation. RNA was harvested from EHTs at 35 days after the initiation of differentiation. RNA extraction was performed using the RNeasy Mini Kit (50) (Qiagen, 74104) according to the manufacturer’s protocol. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) according to the manufacturer’s protocol. RT-qPCR was performed in TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, 4444557) using Taqman probes listed in *Table 4.S1* or PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25741) using primers listed in supplementary *Table 4.S2*. All RT-qPCR reactions were performed according to the manufacturer’s protocol and expression values were normalised to the housekeeping gene *GAPDH*. 10 ng of cDNA was used per reaction. Cycle threshold values were obtained from 3 technical replicates of each sample. Relative gene expression was calculated using the comparative cycle threshold method (Schmittgen and Livak, 2008).

4.2.6 ELISA

4.2.6.1 2D iPSC-CMs

Enzyme-linked immunosorbent assays (ELISAs) were performed using the cell culture supernatant of iPSC-CM for TGF- β 1 (RayBiotech, ELH-TGF β 1-1). 400,000 iPSC-CMs were seeded into each well of 24 well plate 27 days following the initiation of differentiation. The media was changed the following day with 0.5 mL RPMI 1640 supplement 1:50 with

B27 + insulin. Following 48 hours in culture, the cell media was harvested and frozen at -80 °C. The cell culture media was defrosted and the TGF- β 1 inside the supernatant was activated by treating with 1N HCl for 10 minutes, followed by neutralisation with 1.2 N NaOH/0.5 M HEPES. 100 μ l of undiluted but activated cell culture supernatant was used per well of the ELISA. Standard solutions ranging from 125 ng/mL to 16.38 pg/mL TGF- β 1 were prepared and run in duplicate with each ELISA. 100 μ l standard or sample was added to each well of the plate. The plate was incubated for 2.5 hours at room temperature. The supernatant was removed and the plate was washed 3 times. 100 μ l of the biotin antibody was added to each well and the plate was incubated for 1 hour at room temperature. The plate was washed 3 times and 100 μ l of streptavidin solution was added to each well. This was followed by a 45 minute incubation period at room temperature. 3 plate washes were performed prior to the addition of 100 μ l of TMB One-Step Substrate Reagent to each well. The plate was incubated at room temperature for 30 minutes in the dark. 50 μ l of stop solution was added to each well and the absorbance of the plate was read using a spectrophotometer at 450 nm.

4.2.6.2 EHT Samples

ELISA's performed on EHTs were done using supernatant harvested 35 days after the initiation of differentiation (5 days after removal of the TGF- β 1 inhibitor). The media was changed on all the EHTs with 1.6 mL of EHT media (Cumberland et al., 2023). The EHTs were cultured in this media for 48 hour and the supernatant was harvested and frozen at -80 °C. ELISA's were performed on the supernatant for TGF- β 1, Pro-Collagen 1 (R&D Systems, DY6220-05) and Fibronectin. The ELISAs performed to detect TGF- β 1 (RayBiotech, ELH-TGF β 1-1) and Fibronectin (RayBiotech, ELH-FN1-1) in the supernatant of the EHTs was carried out as described in 4.2.6.1. No activation step was required prior to the fibronectin ELISA. The standards solutions were between 150 ng/mL and 0.614 ng/mL.

The Pro-Collagen 1 ELISA was carried out using the DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems, DY008B). Briefly, 96 well plates were coated with capture antibody and incubated overnight at room temperature. The plates were blocked for 1 hour at room temperature using reagent diluent. 100 µl of sample/standard was added to each well and the plate was incubated at room temperature for 2 hours. The plate was washed 3 times prior to the addition of 100 µl of the detection antibody to each well for 2 hours. The plate was washed 3 times and 100 µl of Streptavidin-HRP was added to each well. The plate was incubated in the dark for 20 minutes. The plate was washed 3 times and 100 µl of substrate solution was added to each well. The plate was incubated in the dark for 20 minutes. 50 µl of stop solution was added to each well and the absorbance of the plate was read at 450 nm. The ELISA's used in this study are listed in *Table 4.S3*. All ELISA's were performed according to the manufacturer's protocol.

4.2.7 Immunofluorescence

At day 26 after the initiation of differentiation, iPSC-CMs were dissociated and passaged onto 10 mm geltrex coated coverslips. 4 days later, the cells were fixed in 4 % PFA in PBS for 14 minutes at room temperature. Samples were blocked for 1 hour at room temperature in buffer consisting of 5% Foetal bovine serum, 1% Bovine Serum Albumin (Merck Millipore, A9418-10G) and 0.5% Triton™ X-100 (Merck Millipore, X100-100ML) in PBS. Primary antibodies were added in blocking buffer for 2 hours at room temperature at concentrations detailed in *Table 4.S4*. Samples were washed 3 times in PBS prior to the addition of secondary antibodies for 1 hour at room temperature (covered from light) and at the concentrations detailed in *Table 4.S4*. The nuclei stain DAPI was added with all secondary antibodies at the concentration detailed in *Table 4.S4*. Coverslip samples were washed 3 times in PBS prior to mounting onto cover slides using Hydromount Histology Mounting Media (Scientific Laboratory Supplies, NAT1324). Images were acquired using a Zeiss

LSM780 Confocal laser scanning microscope equipped with a C-Apochromat 63x/1.20 W oil objective.

4.2.8 Sarcomere Analysis

Sarcomere analysis was performed on images acquired from atrial iPSC-CMs 30 days after the initiation of differentiation, stained for α -actinin and phalloidin (F-Actin). Images were acquired from 3 differentiation batches of c.59926+1^{WT/WT} and c.59926+1^{WT/tv} atrial iPSC-CMs. Images were converted into tifs and analysed using the automated z-disc mapping software described in Morris et al., 2020. Mean sarcomere length and orientation order parameter were plotted for each genotype.

4.2.9 Whole-cell patch clamp electrophysiology of Atrial iPSC-CMs

4.2.9.1 Cardiomyocyte Isolation

Atrial iPSC-CMs were dissociated from 6 well plates through treatment with TrypLE™ Select for 30 minutes at 37 °C and passaged into wells of a 24 well plate containing 10 mm coverslips coated according to the manufacturers protocol in geltrex. iPSC-CMs were passaged at 27 days after the initiation of differentiation and cultured in the 24 well plate for a further 3 days prior to patch-clamp analysis. 5×10^4 cells were seeded into each well of the 24 well plate. Coverslips were transferred into a recording chamber and continually superfused with extracellular solution at 3 mL/min⁻¹.

4.2.9.2 Patch-Clamp Recordings

Patch-clamp recordings and analysis were performed using an Axopatch 200B amplifier (Molecular Devices, USA). Analog signals were digitised at 50 kHz using a CED micro1401 operated using Signal v6 software (Cambridge Electronic Design, Cambridge, UK).

4.2.9.3 Voltage Clamp Solutions

Whole cell patch clamp recordings were performed for outward potassium (I_K) and inward sodium current (I_{Na}). I_K was recorded at 37 °C. The internal solution contained (in mM): KCl

135, NaCl 5, EGTA 10, HEPES 10, MgATP 3, Na₃GTP 0.5 and D-glucose 10, pH of 7.2 (adjusted using KOH). The extracellular solution contained (in mM): NaCl 140, KCl 4.5, HEPES 10, D-glucose 10, NiCl₂ 2, MgCl₂ 1.2 and CaCl₂ 1, pH of 7.4 (adjusted with NaOH). I_{Na} was recorded at 37 °C using an internal solution with a final pH of 7.2 (adjusted with CsOH). The solution contained (in mM): CsCl 115, HEPES 10, EGTA 10, MgATP 5, Tetraethylammonium Chloride (TEACl) 20, MgCl₂ 0.5 and NaCl 5. The extracellular solution used in I_{Na} recordings had a final pH of 7.4 (adjusted with CsOH). The solution contained (in mM): NaCl 40, KCl 4.5, Choline Chloride (C₅H₁₄CINO) 100, HEPES 10, D-glucose 10, NiCl₂ 2, MgCl₂ 1.2 and CaCl₂ 1.8. The low sodium solution used in these experiments allowed for sufficient voltage control.

4.2.9.4 Voltage Protocols

Whole-Cell Peak I_{Na} was elicited from a -100 mV holding potential using 100 ms pulses (-100 to +40 mV in 5 mV steps). Steady state inactivation kinetics were assessed using 500 ms pre-pulses from -120 to -40 mV in 5 mV steps, followed by a 50 ms pulse to a test potential of -30 mV. Time-dependent recovery kinetics were assessed using a standard two pulse protocol (-100 mV to -30 mV, 25 ms). The time between the pulses incrementally increased between 1 and 900 ms. Peak I_K was evoked from a -70 mV holding potential using 500 ms pulses to test potentials -70 to +70 mV, in 10 mV increments.

4.2.9.5 Action Potential Measurements in Atrial iPSC-CMs

Spontaneous and stimulated action potentials were recorded from atrial iPSC-CMs at 30 days after the initiation of differentiation. Action potentials were recorded at 37 °C using an internal solution of pH 7.2 (adjusted using KOH). The solution contained (in mM): KCl 135, NaCl 10, MgATP 5, HEPES 10, EGTA 0.1. Extracellular solution contained (in mM): NaCl 145, KCl 5.4, MgSO₄·7H₂O 0.83, NaH₂PO₄ 0.33, HEPES 5, Glucose 11, CaCl₂ 1. Spontaneous action potentials were recorded from cells following a 60 second period after

breakthrough, to allow cell stabilisation. 10 successive spontaneous action potentials were analysed using custom algorithms developed in MatLab. A hyperpolarising current was then applied to cells to hold the diastolic membrane potential at -75 mV prior to recording of stimulated action potentials (Workman et al., 2001) (Holmes et al., 2021). Only those cells that required a hyperpolarising current of less than 150 pA were used (Workman et al., 2001). Action potentials were stimulated using a 1 nA, 2 ms depolarising current injection at a frequency of 1 Hz. 10 successive stimulated action potentials were analysed from each cell.

4.2.9.6 Carbachol Treated Action Potential Recordings

Spontaneous action potentials were recorded for 45 seconds prior to treatment of the cells with the extracellular solution listed above containing 2 μ M carbachol for 2 minutes.

Spontaneous action potentials were subsequently recorded, and the extracellular solution was switched back to one without carbachol. Action potentials were recorded following a further 2 minutes without carbachol to ensure that any changes seen in AP were due to carbachol treatment and not due to loss of seal/cell viability.

4.2.10 Statistical Analysis

All statistics were performed using GraphPad Prism Version 10.0.03. Gaussian distribution was tested using a Shapiro-Wilk normality test. Either an unpaired t-test with a Welch's correction or a mixed-effects one-way ANOVA was used to test significance.

4.2.11 Computer Modelling and Simulation of Human Atrial Electrophysiology

4.2.11.1 *In-silico* Human Atrial CM Models

An *in-silico* population of 1,000 human atrial CM models was generated as in Muszkiewicz et al., (2016) to capture the electrophysiological variability reported in atrial iPSC-CMs. The

population was constructed with a modified version of the CRN model that included I_{KACH} (Courtemanche et al., 1998) (Bayer et al., 2019). Moreover, I_{KACH} formulation was extended to consider the I_{KACH} activation dependent on carbachol (Voigt et al., 2013).

The ionic current densities of the modified CRN model were sampled up to $\pm 50\%$ of their control ranges, using Latin Hypercube sampling. The resulting 1,000 CM models underwent calibration against action potential biomarkers derived from human patients in sinus rhythm and AF (Sánchez et al., 2014). Thus, from the original population, 830 atrial CM models presented action potential biomarkers in range with the human data and were used to analyse the effects of $2 \mu\text{M}$ carbachol, as conducted experimentally with atrial iPSC-CMs.

4.2.11.2 In-silico Human Whole-atria Models

Two atrial CM models reproducing the experimental conditions for control and *TTN*_{tv} were chosen to conduct multi-scale simulations, using the detailed whole-atria model described in (Ferrer et al., 2015). The virtual atria include a realistic location of ganglionated plexuses, allowing simulation of a heterogeneous acetylcholine release in the atria (Celotto et al., 2022).

The single-cell properties of the atrial CM model were included in the right atrial tissue and were scaled in the left atrium, crista terminalis, pectinate muscles, left atrial appendage and atrio-ventricular rings, according to reported regional differences on ion channel expression (Dasí et al., 2022).

Sinus rhythm simulations were conducted considering a maximum release of $0.1 \mu\text{M}$ acetylcholine in ganglionated plexus sites (Celotto et al., 2022).

4.3 Results

4.3.1 Generation of iPSCs carrying the clinically relevant TTN c.59926+1 G>A mutation

An AF-associated A-band TTNtv (*TTN* c.59926+1; *Figure 4.1A*) was introduced into a healthy iPSC line by CRISPR/Cas9 technology. A HDR donor containing the TTNtv and *HindIII* site was incubated with the *TTN* c.59926+1 targeting CRISPR/Cas9 complex prior to transfection (*Figure 4.S3*). Clonal establishment was performed by low-density culture where the clonal genotype was analysed by on-PCR restriction digestion (*Figure 4.1B*). *HindIII* digestion of genomic PCR product (637 bp) encompassing the TTNtv/*HindIII* sites allowed identification of both heterozygous (WT, 637 bp; TTNtv, 280 and 357 bp) and homozygous TTNtv lines (TTNtv only, 280 and 357 bp). Subsequently, the genotypic status was verified by DNA sequencing (*Figure 4.1C*). Both heterozygous (*TTN* c.59926+1^{WT/tv}) and homozygous (*TTN* c.56626^{tv/tv}) lines were confirmed for expression of the pluripotency markers: OCT3/4, SOX2, SSEA4 and NANOG (*Figures 4.S1*) and normal karyotype (*Figure 4.S2*). Wild type iPSCs were differentiated into atrial iPSC-CMs and demonstrated compartment specific electrophysiological, genetic, and structural markers (*Figure 4.S4*).

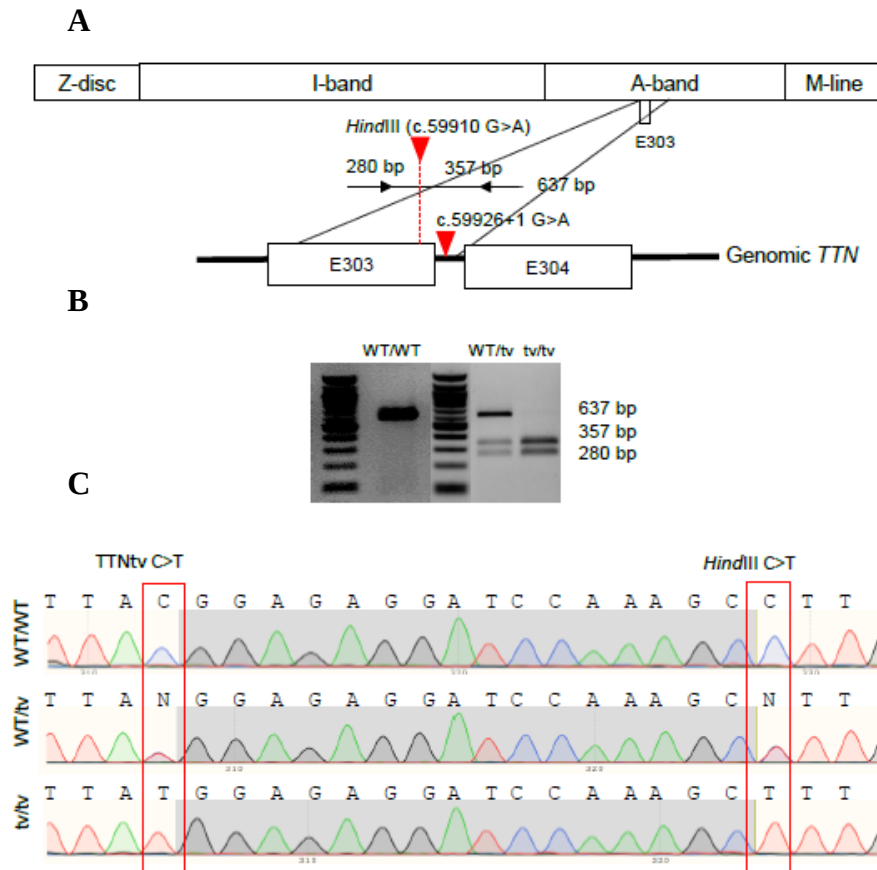


Figure 4.1: CRISPR/Cas9-assisted Generation of TTNtv iPSC lines

(A) The A-band TTNtv (*TTN* c.59926+1 G>A, located within the intron immediately downstream of exon 303) together with the silent mutation corresponding to a HindIII site (*TTN* c.59910 G>A) were introduced into an iPSC line by CRISPR/Cas9 targeting technique. A PCR strategy covering both the mutation sites (637 bp) was applied to genotype isolated iPSC colonies guided by the digestion pattern of HindIII enzyme (280 and 357 bp). (B) The wild-type PCR product (WT/WT) cannot be digested by HindIII whilst successful genome editing resulted in cleavage of the PCR amplicon. The assay allowed identification of both heterozygous (WT/tv; incomplete digestion) and homozygous clones (tv/tv; complete digestion). (C) Confirmation of the genomic changes was demonstrated by Sanger DNA sequencing. The chromatogram presented was derived from the anti-sense strand in the 5' to 3' direction as read from the left to the right.

4.3.2 Sarcomere Integrity and *TTN* Gene Expression

Atrial iPSC-CMs carrying each of the 3 *TTN* genotypes (c.59926+1^{WT/WT}, c.59926+1^{WT/tv} and c.59926+1^{tv/tv}) were differentiated from iPSCs and matured for 30 days. Atrial iPSC-CMs derived from the wild type (*TTN* c.59926+1^{WT/WT}) and heterozygous (*TTN* c.59926+1^{WT/tv}) cell lines formed/displayed functional and organised sarcomeres, as demonstrated by the α -actinin staining shown in Figure 4.2A. Atrial CMs differentiated from the homozygous (*TTN* c.59926+1^{tv/tv}) line were unable to form functional sarcomeres and consequently showed no contractile activity. *TTN* RNA splicing was analysed by RT-PCR using DNA probes

spanning exons 303 and 304. The *TTN* c.59926+1^{WT/WT} cells exclusively transcribed the expected 552-bp transcript belonging to the WT *TTN* species (Figure 4.2B). The *TTN* c.59926+1^{WT/tv} cells expressed the WT transcript and a slightly larger mutant *TTN* species isoform. The *TTN* c.59926+1^{tv/tv} exclusively produced the mutant *TTN* transcript. Sequence analysis of the mutant *TTN* cDNA indicated retention of the intron (85 bp) immediately downstream of exon 303, which is predicted to contain a premature stop codon (*TTN* p.H10911RfsX16 in the cardiac N-2B isoform (Figure 4.S5), thereby truncating the protein. RT-qPCR of exon junctions spanning different regions of the *TTN* gene demonstrated a broad downregulation in *TTN* gene expression (Figure 4.2C). The *TTN* c.59926+1^{tv/tv} cells were not used throughout the rest of the study due to limited clinical relevance and their innate inability to form functional sarcomeres or to contract.

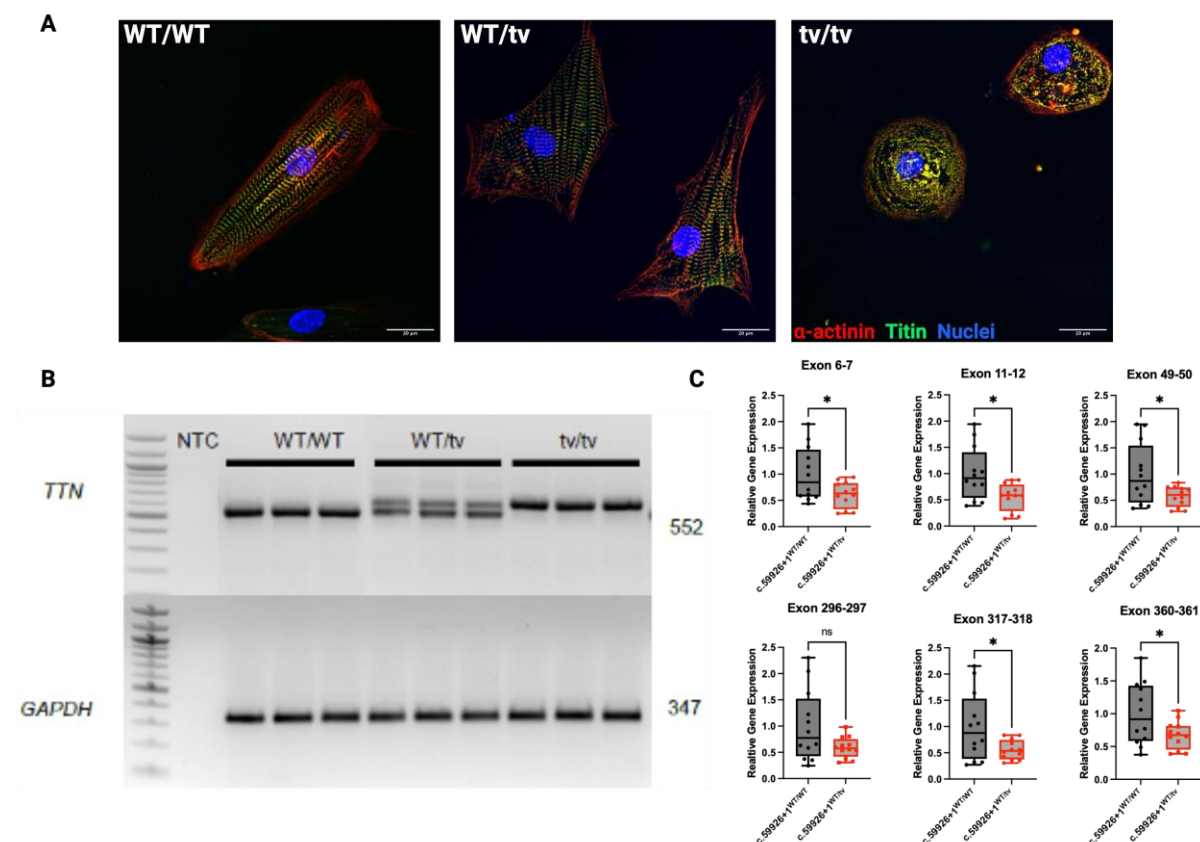


Figure 4.2: Differential Sarcomere Formation and *TTN* Gene Expression in c.59926+1^{WT/WT}, c.59926+1^{WT/tv} and c.59926+1^{tv/tv} atrial iPSC-CMs

(A) Day 30 iPSC-CMs were fixed and stained for α -actinin, titin (z-disc region) and DAPI (nuclei). (B) RT-PCR was performed using primers that annealed at exons 303 and 304. The mutant *TTN* product can be differentiated from the WT by its slightly larger size (>552 bp). (C) Day 30 Atrial iPSC-CMs were harvested for RNA. Following cDNA conversion, qPCR was performed using primers that amplified different regions of the *TTN* transcript (N = 4 Differentiation Batches, n = 12 Technical Replicates). Gene expression was normalised to *GAPDH*. A Welch's t test was used to ascertain significance (* denotes $P < 0.05$). Scale bar = 20 μ m.

4.3.3 Atrial iPSC-CM Sarcomere Length and Cardiac Stress

To further explore the impact the truncating variant was having on sarcomere organisation, day 30 atrial iPSC-CMs were fixed and stained for α -actinin, F-actin and DAPI. Images of the cells were analysed using a Z-line detection script described in Morris et al., (2020). The *TTN* c.59926+1^{WT/tv} cells formed similarly organised ($P = 0.94$) but shortened sarcomeres ($P < 0.0001$) (Figure 4.3A). A panel of cardiac stress gene markers was assayed using RT-qPCR. Analysis performed on the atrial iPSC-CMs revealed an upregulation in the gene expression of the pro-fibrotic cardiac stress markers: platelet derived growth factor A (*PDGFA*) ($P = 0.0051$) and platelet derived growth factor B (*PDGFB*) ($P = 0.0013$) (Figure 4.3B). Subsequently, gene expression and protein secretion of the pro-fibrotic cytokine transforming growth factor β 1 (TGF- β 1) was assessed using RT-qPCR and ELISA. The cells demonstrated no significant change in TGF- β 1 gene expression or protein secretion (Figure 4.3C).

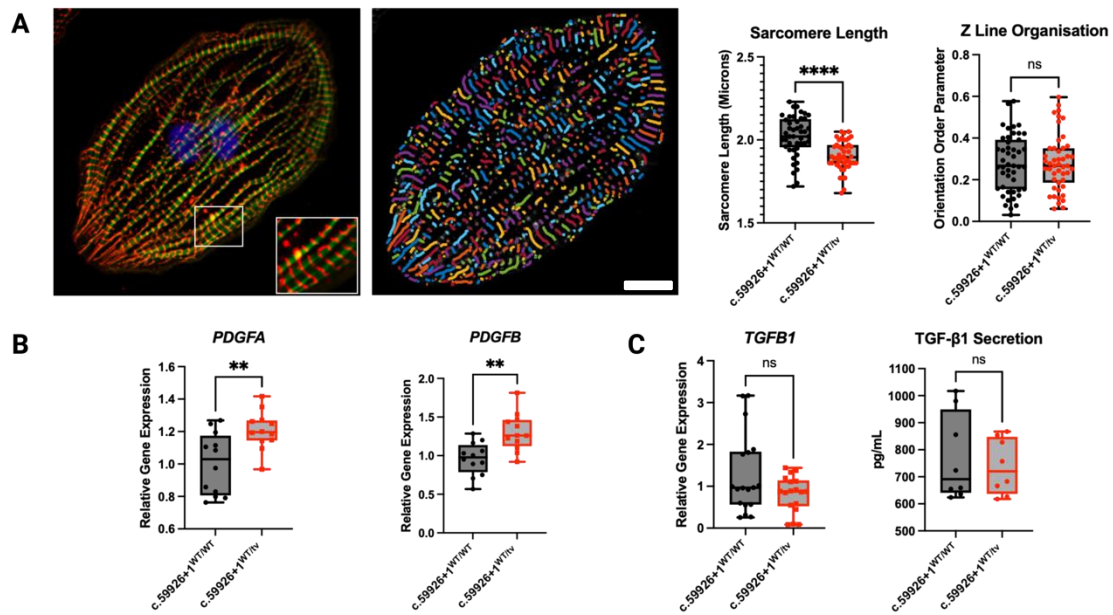


Figure 4.3: Sarcomere Shortening and Augmented Cardiac Stress in *TTN* c.59926+1^{WT/tv} Atrial iPSC-CMs

(A) Top Left: iPSC-derived atrial cardiomyocytes were fixed and stained for α -actinin, F-actin and DAPI (Nuclei) Scale bar = 10 microns. Top Right: The Z-lines of the cells were mapped and analysed using a Z-line detection script (Morris et al., 2020) to ascertain sarcomere length and organisation (orientation order parameter). *TTN* c.59926+1^{WT/WT} $N = 3$, $n = 50$, *TTN* c.59926+1^{WT/tv} $N = 3$, $n = 47$. A Welch's t test was used to ascertain significance (**** denotes $P < 0.0001$). (B) Day 30 atrial iPSC-CMs were harvested for RNA. Following cDNA conversion, qPCR was performed to quantify the expression level of cardiac stress markers *PDGFA* and *PDGFB* ($N = 4$, $n = 12$). Gene expression was normalised to *GAPDH*. A Welch's t test was used to ascertain significance (** denotes $P < 0.01$). (C) Day 30 atrial iPSC-CMs were harvested for RNA (cell pellets) and secreted protein (supernatant). Following cDNA conversion, the expression of pro-fibrotic cytokine TGF- β 1 was measured by qPCR ($N = 6$, $n = 18$). A Welch's t test was used to ascertain significance (ns = $P > 0.05$). An ELISA was performed on the supernatant to assess secretion of TGF- β 1 ($N = 4$, $n = 8$). A Welch's t test was used to ascertain significance (ns denotes $P > 0.05$).

4.3.4 Electrophysiological Analysis of Atrial iPSC-CMs

To explore the impact the titin variant had on the atrial cellular electrophysiology, spontaneous and stimulated action potentials were recorded from *TTN* c.59926+1^{WT/WT} and *c.59926+1^{WT/tv}* atrial iPSC-CMs. Spontaneous action potential frequency was increased in cells carrying the *TTN* c.59926+1^{WT/tv} variant ($P = 0.048$) (Figure 4.4A). RT-qPCR analysis showed an increase in the expression of the ion channel subunit genes *KCNJ3*, *KCNK1* and *KCND2* in the *TTN* c.59926+1^{WT/tv} cells (Figure 4.4B). Of these, *KCNJ3* has been linked with AF (Yamada et al., 2019). Baseline AP morphology was not different between

genotypes, including stimulated and spontaneous AP morphology (Figure 4.S6 & 6.S7). Measurements of peak voltage dependent I_{Na} (Figure 4.S8) and peak voltage dependent I_K (Figure 4.S9) were consistent between genotypes. Welch's t-tests on the biophysical parameters of sodium channel activity revealed no significant difference in the I_{Na}^+ density, V_{50} activation, k activation, V_{50} inactivation and k inactivation (Table 4.S5). The I_{Na} time recovery P50 was significantly lower in the TTN c.59926+1^{WT/tv} cells, which may partially explain the increased beating frequency.

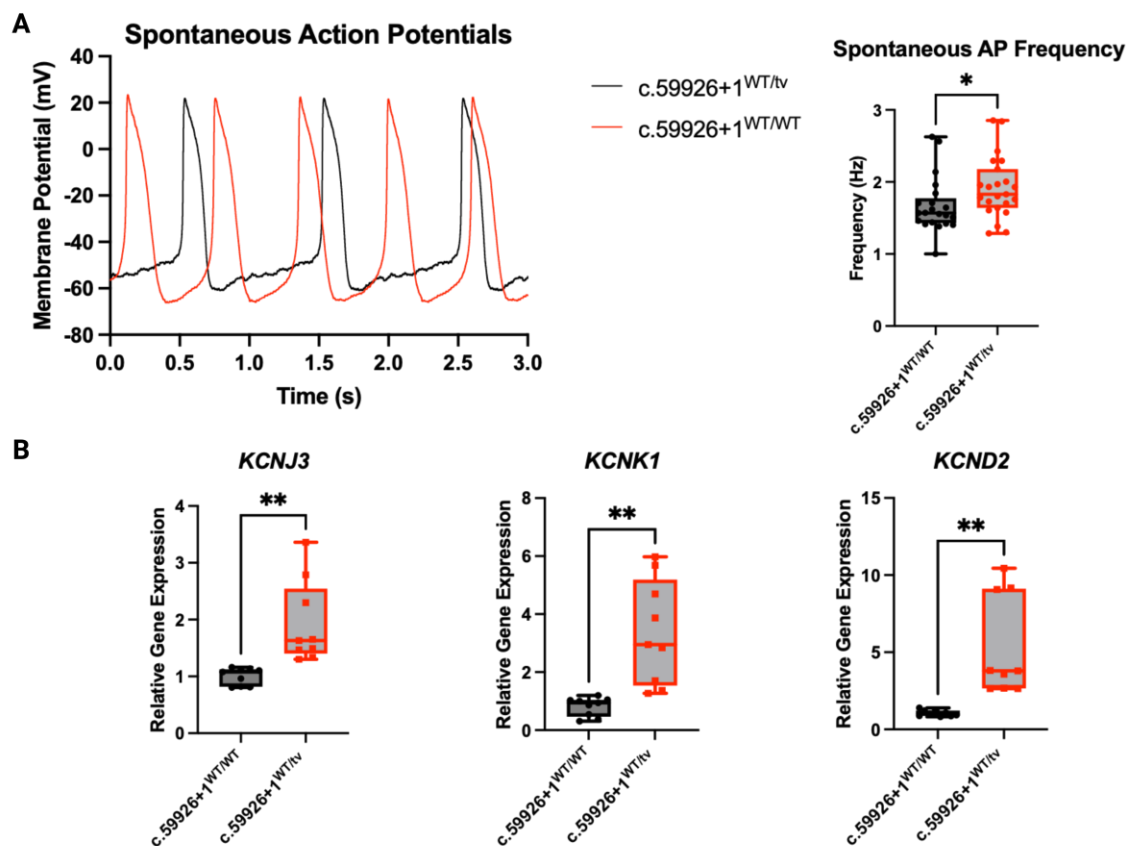


Figure 4.4: Alterations in Spontaneous Action Potential Frequency and Ion Channel Subunit Gene Expression in TTN c.59926+1^{WT/tv} atrial iPSC-CMs

Patch clamp analysis of atrial iPSC-CMs was performed 30 days after initiation of differentiation. (A) Top Left: A representative spontaneous action potential trace from TTN c.59926+1^{WT/WT} and TTN c.59926+1^{WT/tv} cells. Top Right: Spontaneous action potential frequencies observed in TTN c.59926+1^{WT/WT} ($N = 3$, $n = 21$) and c.59926+1^{WT/tv} cells ($N = 3$, $n = 23$). A Welch's t test was used to ascertain significance (* denotes $P < 0.05$). (B) Day 30 Atrial iPSC-CMs were harvested for RNA. Following cDNA conversion, qPCR was performed using primers designed to amplify ion channel subunit genes $KCNJ3$, $KCNK1$ and $KCND2$ ($N = 3$, $n = 12$). A Welch's t test was used to ascertain significance (** denotes $P < 0.01$).

4.3.5 Susceptibility of Atrial iPSC-CMs to Acetylcholine Agonist Carbachol

The ion channel subunit gene *KCNJ3* encodes the G protein-activated inward rectifier potassium channel 1 (GIRK1/K_{ir}3.1) which combines with G protein-activated inward rectifier potassium channel 4 (GIRK4/K_{ir}3.4) to regulate the acetylcholine-sensitive K⁺ current, $I_{K_{ACh}}$ in sinoatrial and atrial CMs. Gain of function mutations in *KCNJ3*, which increase activity of GIRK1/4, have been linked to a dysregulation in heart rate and an increased prevalence of AF (Yamada et al., 2019). Here, spontaneous action potentials were recorded from *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} atrial iPSC-CMs with and without carbachol treatment (a $I_{K_{ACh}}$ agonist). Representative action potential traces from *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} are shown in the presence and absence of carbachol in *Figure 4.5A*. The action potential duration at 90 % repolarisation (APD90) was reduced in both groups following carbachol treatment, however this was significantly exaggerated in *TTN* c.59926+1^{WT/tv} iPSC-CMs (*Figure 4.5A & B*). Carbachol treatment elicited no significant effect on the action potential amplitude in *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} cells and caused a similar mild hyperpolarisation of the diastolic membrane potential in both genotypes (*Figure 4.5B*).

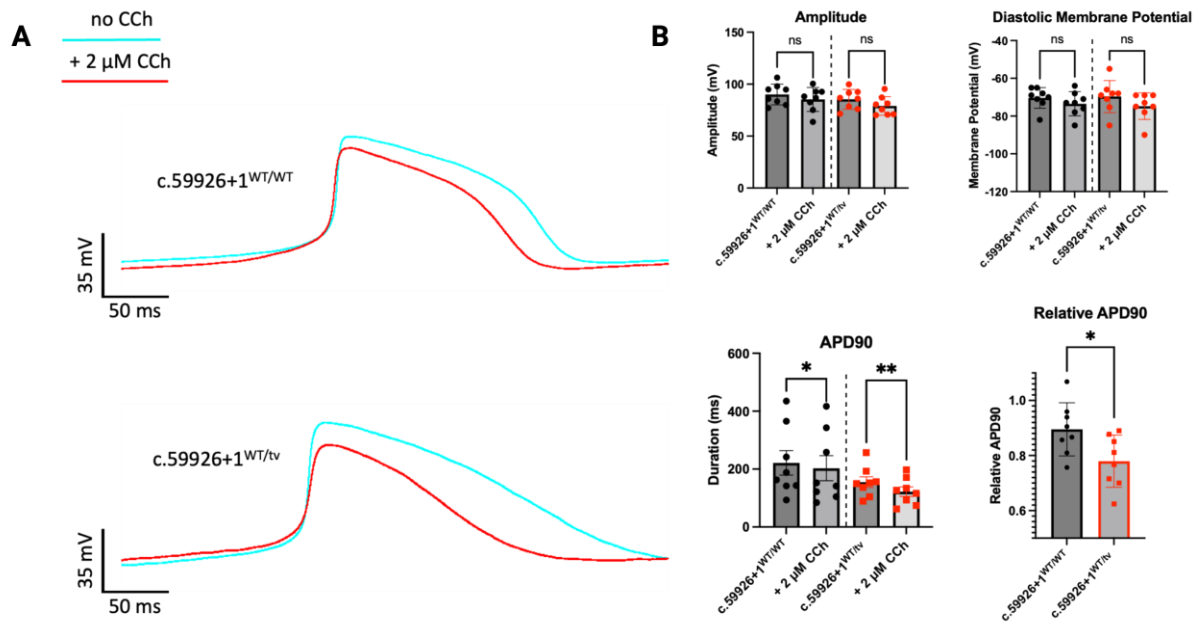


Figure 4.5: Enhanced electrophysiological sensitivity to carbachol in TTN c.59926+1^{WT/tv} atrial iPSC CMs

Patch clamp analysis of atrial iPSC-CMs was performed 30 days after initiation of differentiation. Spontaneous action potentials were recorded prior (blue) to and after the treatment (red) of the cells with 2 μ M of the acetylcholine agonist carbachol. (A) Representative traces of spontaneous action potentials recorded prior to and after the addition of 2 μ M carbachol in TTN c.59926+1^{WT/WT} and TTN c.59926+1^{WT/tv} atrial iPSC-CMs. (B) The effect carbachol treatment has on AP amplitude, diastolic membrane potential, APD90 and relative APD90 post carbachol treatment (N = 3, n = 8). A mixed-effects one-way ANOVA (Šidák correction) was performed to ascertain significance of carbachol treatment. A Welch's t test was used to ascertain significance of relative APD90s following treatment (*p < 0.05, ** p < 0.01, *** p < 0.001).

4.3.6 Susceptibility of in-silico Atrial CMs to Acetylcholine Agonist Carbachol

The sensitivity of atrial CMs to the acetylcholine agonist carbachol was further analysed *in-silico*. Figure 4.6A shows the action potential traces of the initial and calibrated population of atrial CM models, and Figure 4.6B illustrates the effects of 2.0 μ M carbachol on the calibrated population.

The calibrated population of 830 atrial CMs was split in two sub-groups: those representative of TTN c.59926+1^{WT/WT} and those of c.59926+1^{WT/tv} cells. Atrial CM models forming the wildtype subgroup presented a I_{KACH} density equal or lower than the baseline (i.e., scaling factor ≤ 1). According to the experiments, the TTN c.59926+1^{WT/tv} atrial iPSC-CMs presented a 70% increase in *KCNJ3* relative gene expression compared to the TTN c.59926+1^{WT/WT} cells. Therefore, the heterozygous subgroup included atrial CM models with a I_{KACH} density

equal or higher than 70% (i.e., scaling factor ≥ 1.7). These two in-silico subpopulations additionally showed the greater APD shortening occurring for the heterozygous subgroup in response to 2.0 μM carbachol (Figure 4.6C). This enhanced sensitivity to cholinergic APD shortening was even more apparent when simulated in the presence of acetylcholine (ACh; 0.1 μM) (Figure 4.6D).

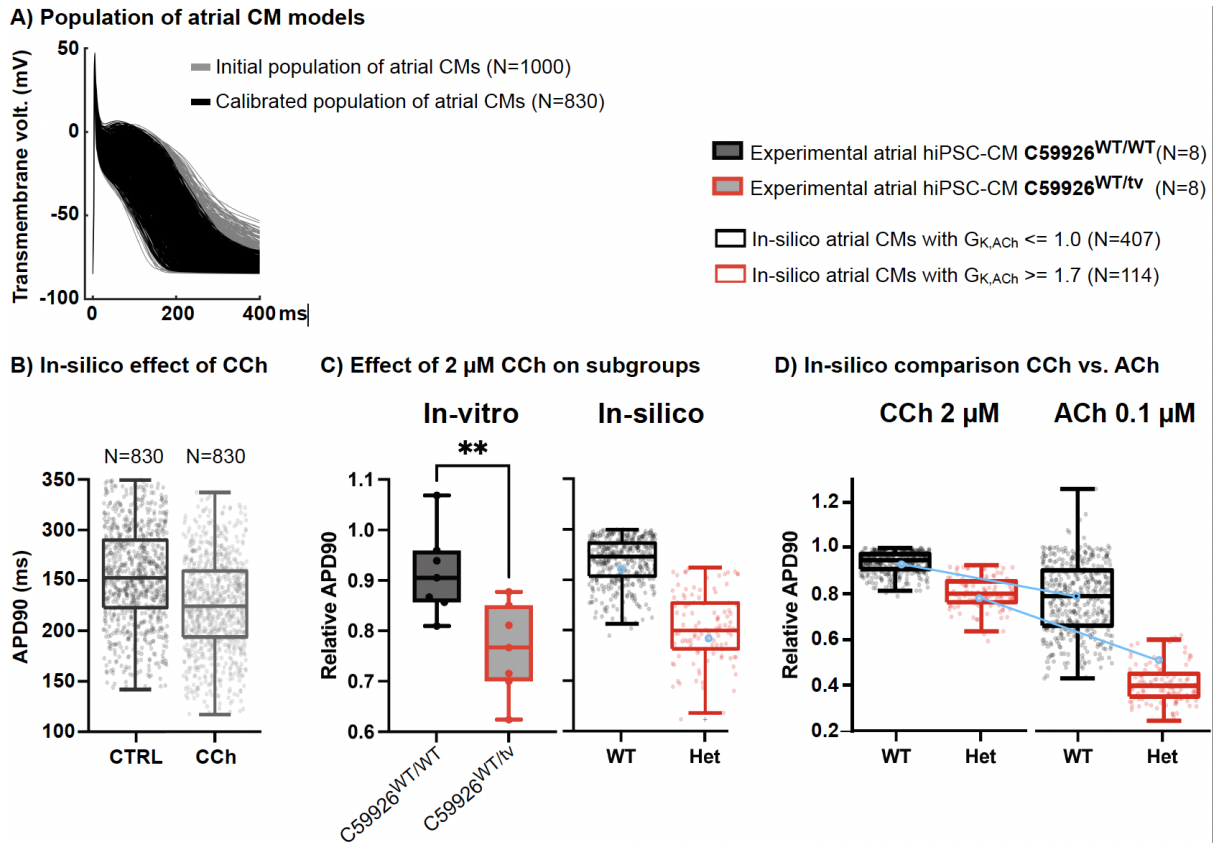


Figure 4.6: In-silico Action Potential Properties Following Carbachol Simulation

Initial and calibrated population of atrial cardiomyocyte (CM) models (A). Action potential duration at 90% of cellular repolarisation (APD90) for control conditions (CTRL) and after 2.0 μM carbachol (CCh) in the calibrated population of atrial CM models (B). Comparison of the relative APD90 (prior and after 2.0 μM CCh) in-vitro vs. in-silico for TTN c.59926+1^{WT/WT} and c.59926+1^{WT/tv} atrial iPSC-CM (in-vitro) and atrial CM models with baseline (≤ 1 , WT) or up-regulated (≥ 1.7 , Het) $I_{K\text{ACh}}$ density (in-silico) (C). In-silico comparison of the effects of 2.0 μM CCh vs. 0.1 μM acetylcholine (ACh) for the same subgroups shown in (C). (D) The blue circle highlights the atrial CM model used for multi-scale simulations.

4.3.7 TTNtv creates a heterogeneous repolarisation substrate sufficient to precipitate AF *in-silico*

Two atrial CM models, one for wild-type and one for the heterozygous condition, presenting the same relative APD (i.e., ratio between control and 2.0 μM of carbachol) as the median

observed for c.59926+1^{WT/WT} (i.e., ≈ 0.9) and c.59926+1^{WT/tv} (i.e., ≈ 0.8) were used for multi-scale simulations (*Figure 4.6C*).

When these atrial CMs were exposed to 0.1 μ M ACh, the relative APD decreased to 0.79 and 0.55 for c.59926+1^{WT/WT} and c.59926+1^{WT/tv}, respectively (*Figure 4.6D* and *Figure 4.7 top*).

Thus, the release of 0.1 μ M ACh by the ganglionated plexuses, and the subsequent APD shortening at these sites, created a heterogeneous repolarisation substrate (*Figure 4.S10*). The heterogeneous repolarisation was exaggerated in simulated c.59926+1^{WT/tv} conditions, due to I_{KACH} up-regulation and the greater APD difference between acetylcholine-release sites (i.e., ganglionated plexus) and the bulk tissue (*Figure 4.S10* and *Figure 4.7*).

In the event of sinus tachycardia, or only an extra beat applied 200 ms after the preceding beat, the heterogeneous repolarisation substrate seen in c.59926+1^{WT/tv} was sufficient to evoke AF even in the absence of structural remodelling (*Figure 4.7B*). Under the same stimulation conditions, AF was not induced in c.59926+1^{WT/WT} (*Figure 4.7A*)

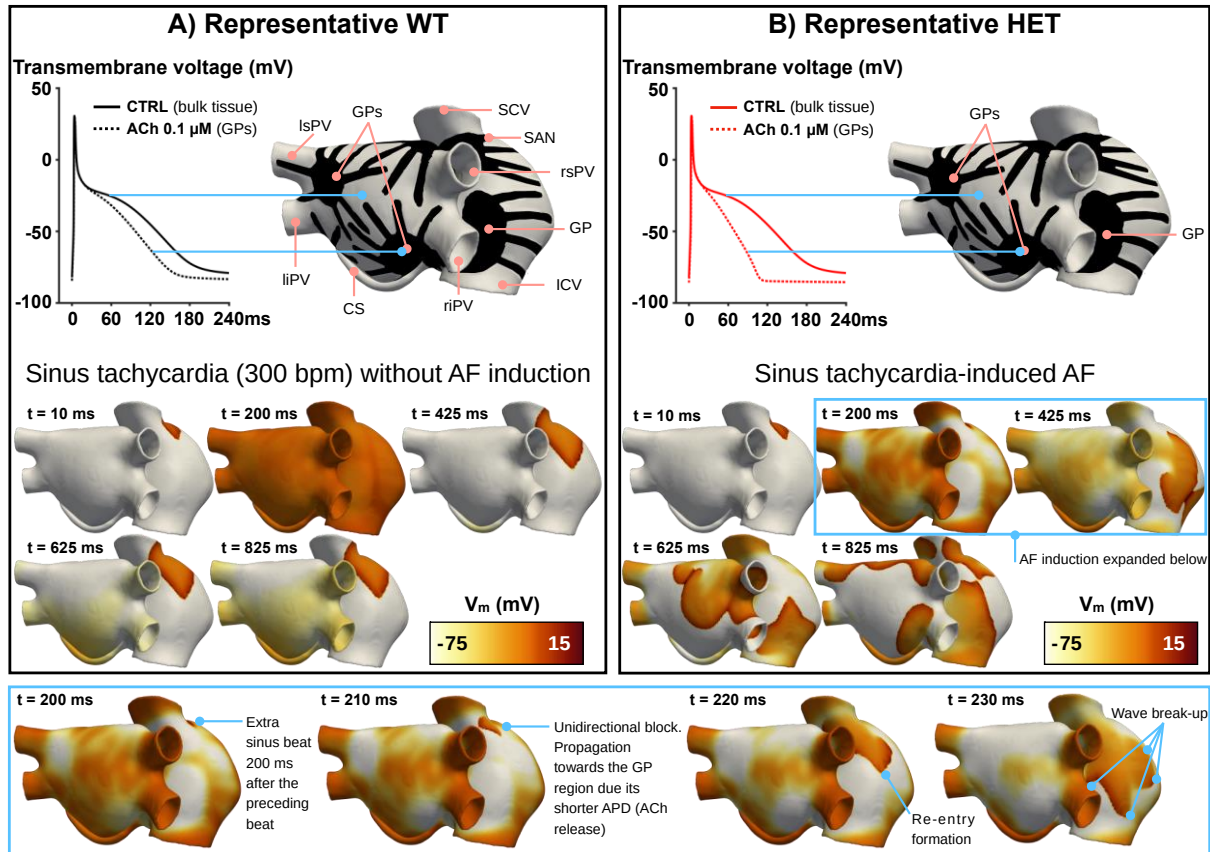


Figure 4.7: Sinus tachycardia-induced AF for heterozygous *TTN*tv (HET) but not wild-type (WT) conditions

A: Atrial cardiomyocyte models used for WT and a schematic representation of the human whole-atria model used in the multi-scale simulations. Consecutive snapshots of the atrial transmembrane voltage (V_m) showing that, under wild-type conditions, sinus tachycardia (i.e., 5 beats applied at a cycle length of 200 ms) is unable to evoke AF. **B:** Atrial cardiomyocyte models used for heterozygous *TTN*tv (HET) conditions. Snapshots of V_m showing AF induction and maintenance. The initiation of AF for the heterozygous conditions is illustrated and explained in the blue box. Abbreviations. SAN: sinoatrial node; SCV-ICV: superior and inferior cava vein; rs-ri-ls-li-PV: right superior, right inferior, left superior and left inferior pulmonary vein; CS: coronary sinus. The release of 0.1 μ M acetylcholine only occurs at the ganglionated plexuses (GPs), arranged following an octopus configuration.

4.3.8 Assessing Structural Remodelling in Co-Culture Engineered Heart Tissues

Overexpression of *PDGFA* and *PDGFB* is linked to structural remodelling and cardiac fibrosis. Co-cultured engineered heart tissues (EHTs) were established to assess the effect the *TTN* c.59926+1^{WT/tv} variant may have on cellular crosstalk. EHTs were constructed using *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} atrial iPSC-CMs and quiescent iPSC-CFs (Figure 4.8A) (Cumberland et al., 2023). The *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} EHTs showed no significant difference in time to peak, contraction amplitude, relaxation time, contraction duration and peak to peak time, as demonstrated in Figure 4.S11. However, the *TTN* c.59926+1^{WT/tv} EHTs had a significant upregulation of the profibrotic and extracellular

matrix genes: *PDGFB*, *COL1A1* and *COL3A1* (Figure 4.8B). ELISA's performed on the supernatant identified a significant increase in the secretion of the pro-fibrotic cytokine TGF- β 1 and the extracellular matrix proteins: Pro-Collagen 1 and Fibronectin-1 (Figure 4.8C).

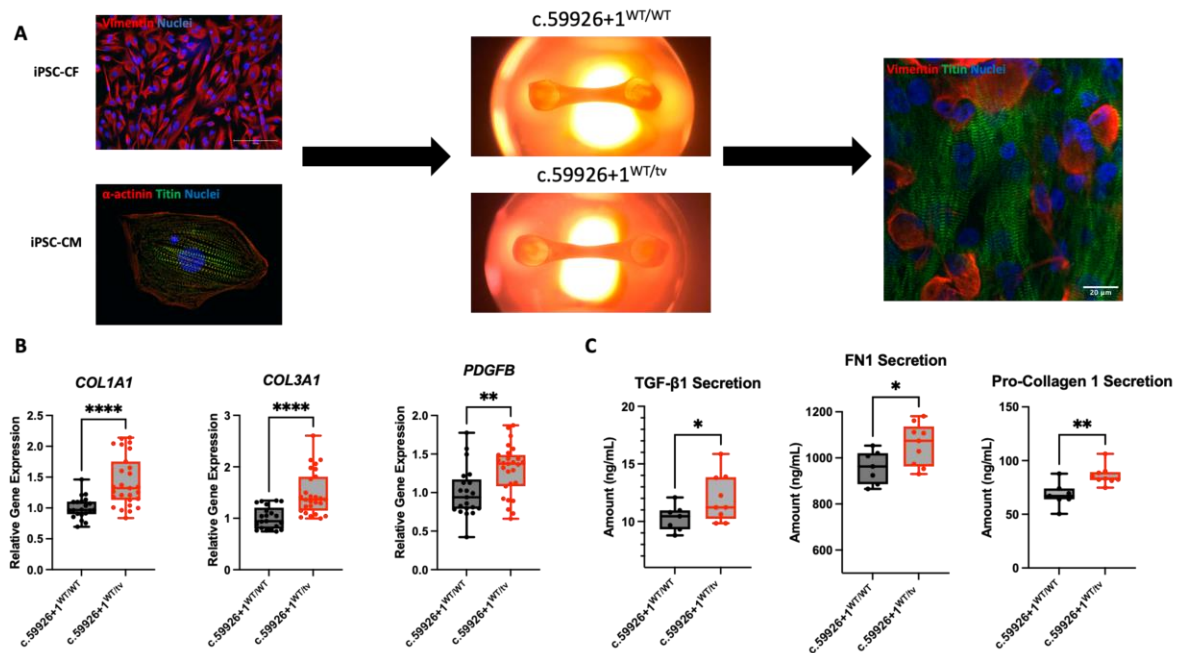


Figure 4.8: Pro-fibrotic Gene Expression and Structural Remodelling in *TTN* c.59926+1^{WT/tv} EHTs

Co-cultured EHTs constructed using atrial iPSC-CMs and quiescent iPSC-CFs were matured to day 30 after the initiation of differentiation (A). RNA was extracted from EHTs and RT-qPCR was performed on EHTs for pro-fibrotic and extracellular matrix genes: *COL1A1*, *COL3A1* and *PDGFB* (N = 3 batches of EHTs, n = 7 EHTs for *TTN* c.59926+1^{WT/WT} and n = 9 EHTs for *TTN* c.59926+1^{WT/tv}, 3 technical replicates performed per EHT) (B). Supernatant was taken from the EHTs and ELISA's were performed to ascertain levels of TGF- β 1, FN-1 and Pro-Collagen 1 (C). A Welch's t test was used to ascertain significance (* denotes $P < 0.05$, ** denotes $P < 0.01$, **** denotes $P < 0.0001$).

4.4 Discussion

Incorporation of the AF associated *TTN* c.59926+1 variant into iPSCs was achieved using the CRISPR/Cas9 engineering tool (*Figure 4.1*). To the best of our knowledge, this is the first study to assess the pathological effect a *TTN*tv has in atrial iPSC-CMs. The heterozygous iPSC-CMs demonstrated an increased propensity for cardiac fibrosis and structural remodelling, evidenced by increased expression and secretion of extracellular matrix proteins. Increased spontaneous beating frequencies and susceptibility to the acetylcholine agonist carbachol indicate signs of pro-arrhythmic electrical remodelling.

In agreement with similar studies assessing different *TTN*tv, homozygous *TTN* c.59926+1^{tv/tv} atrial iPSC-CMs failed to construct organised or functional sarcomeres (*Figure 4.2A*) (Hinson et al., 2015). Homozygous *TTN*tv identified in patients are extremely rare. Furthermore, numerous *in vivo* studies indicate that most homozygous *TTN*tv cause *in vivo* embryonic lethality (Huttner et al., 2018) (Akinrinade et al., 2015) (Gramlich et al., 2009) (Schafer et al., 2017) (Ahlberg et al., 2018). Therefore, the severe impairment of sarcomerogenesis in homozygous *TTN* c.59926+1^{tv/tv} meant that it was not considered an adequate model to assess *TTN*tv associated AF with the heterozygous *TTN* c.59926+1^{WT/tv} instead offering a better representation of the clinical phenotype. Introduction of the putative splice site mutation into iPSCs caused retention of the distally located downstream intron, which carries a premature stop codon (*Figure 4.2B*). This likely caused the decreased *TTN* gene expression observed in *TTN* c.59926+1^{WT/tv} atrial iPSC-CMs (*Figure 4.2C*) (Schafer et al., 2017).

Z-disc analysis of *TTN* c.59926+1^{WT/WT} and c.59926+1^{WT/tv} atrial iPSC-CMs demonstrated a significant reduction in the sarcomere length of the heterozygous cells, but otherwise unchanged organisation (*Figure 4.3A*). This is in agreement with previous studies investigating other *TTN*tv in adult CMs and ventricular iPSC-CMs and implicates substantial incorporation and function of the truncated titin isoform in the sarcomere (Fomin et al., 2021)

(McAfee et al., 2021) (McAfee et al., 2023) (Hinson et al., 2019). The decreased sarcomere length accompanied an increase in expression of the cardiac stress marker ligand genes *PDGFA* and *PDGFB* in *TTN* c.59926+1^{WT/tv} atrial iPSC-CMs. *PDGFA* and *PDGFB* encode chains that dimerise to form heparin-binding growth factors. These growth factors are integral to heart development and are capable of interacting with a broad range of mesoderm-derived cells, including cardiac fibroblasts (Raica and Cimpean, 2010). Activation and overexpression of PDGF receptors and ligands is often induced by mechanical stress and is associated with the development of atrial fibrosis, AF and arrhythmogenic cardiomyopathies (Ma et al., 1999) (Lee et al., 2019) (Chen et al., 2022) (Jiang et al., 2016).

Although the majority of electrical function is preserved in *TTN* c.59926+1^{WT/tv}, we identified some important signs of electrical remodelling and changes in ion channel gene expression. In agreement with the reported increased prevalence of atrial and ventricular tachycardias in patients with *TTN*tv, the spontaneous action potential frequencies of the *TTN* c.59926+1^{WT/tv} iPSC-CMs were significantly greater (*Figure 4.4A*) (Corden et al., 2019) (Ahlberg et al., 2018) (Hoorntje et al., 2018). Gene expression analysis demonstrated an upregulation of *KCNK1*, *KCND2* and *KCNJ3* (*Figure 4.4B*). *KCNK1* encodes the two-pore domain potassium channel TWIK-1 which plays a role in maintaining a normal heart rate and atrial morphology (Christensen et al., 2016). It has been reported that knockdown of *KCNK1* evokes a significant bradycardia and so, it is possible that the overexpression of *KCNK1* is responsible for the increased action potential frequency observed in the *TTN* c.59926+1^{WT/tv} atrial iPSC-CMs.

A recent study by Huang et al., (2023) demonstrated increased expression of *KCNJ3* in a different ventricular *TTN*tv iPSC-CM model, suggesting that this is a consistent modification associated with various *TTN*tv. Treatment of atrial iPSC-CMs with the acetylcholine agonist carbachol revealed an increased sensitivity of the *TTN* c.c.59926+1^{WT/tv} iPSC-CM, as

evidenced by a significantly greater reduction in APD (*Figure 4.5*). Increased shortening of the APD in response to ACh release during cholinergic stimulation likely causes a heterogeneous repolarisation substrate conducive of AF in patients with c.59926+1G>A TTNtv, as shown by our simulations (*Figure 4.7*).

Atrial dilatation and fibrosis are the first symptoms to manifest in TTNtv animal models (Ahlberg et al., 2018) (Huttner et al., 2018). This has led members of the field to consider whether the pathology of TTNtv can be described as an atrial cardiomyopathy (Fatkin et al., 2019) (Vad et al., 2020). Co-cultured EHTs constructed from *TTN* c.59926+1^{WT/tv} atrial iPSC-CMs exhibited no significant deficit in contractile function when compared to their *TTN* c.59926+1^{WT/WT} counterparts (*Figure 4.S11*). Despite this, the *TTN* c.59926+1^{WT/tv} EHTs displayed a significant upregulation of the pro-fibrotic gene *PDGFB* and the extracellular matrix genes: *COL1A1* and *COL3A1* (*Figure 4.8B*). This corresponded with increased secretion of the pro-fibrotic cytokine TGF-β1, and the extracellular matrix proteins fibronectin-1 and pro-collagen 1 (*Figure 4.8C*). Overexpression of *PDGFA* and *PDGFB* in cardiomyocytes has previously been shown to cause cardiac fibrosis and structural remodelling through paracrine activation of cardiac fibroblasts through PDGF receptors (PDGFR) α and -β (Gallini et al., 2016). Therefore, it is likely that the increased secretion of TGF-β1 in co-culture is due to the pathological interactions between atrial iPSC-CMs and iPSC-CFs, with *PDGFB* acting as the mediator in cardiac fibroblast activation.

Approximately 40 % of the individuals with the mutation in the Hoorntje et al., (2018) study demonstrated atrial dilatation, implicating structural remodelling of the atria as an significant mechanism in the pathology of the variant. Disruption to signal propagation caused by cardiac fibrosis is a common feature of AF (Everett and Olgin, 2007) and our data suggests that this is likely to be a prominent mechanism underlying AF associated with *TTN*

c.59926+1 G>A. This opens up the possibility of pharmacological intervention, with PDGFR inhibitors already established as a strategy for mitigating cardiac fibrosis (Wang et al., 2017).

4.4.1 Conclusions

The *TTN* c.59926+1 G>A variant caused a broad downregulation of total *TTN* gene expression and shortened sarcomeres, which likely confers cardiac stress and increased expression in the pro-fibrotic genes *PDGFA* and *PDGFB*. Although demonstrating broadly preserved intracellular electrophysiology, the variant conferred disruption to heart rate regulation and increased sensitivity to carbachol mediated by overexpression of the acetylcholine dependent G-protein inward rectifier potassium channel subunit gene *KCNJ3*. In co-culture EHTs, the c.59926+1^{WT/tv} *TTN*tv caused increased production of extracellular matrix proteins, which indicates a role for atrial fibrosis in the pathophysiology. Exaggerated fibrosis, higher spontaneous atrial rates and heterogeneous APD shortening in response to cholinergic stimulation likely combines to initiate and maintain AF in patients carrying the c.59926+1 G>A *TTN*tv, as shown by our simulations.

This comprehensive study not only sheds light on the structural and electrophysiological consequences of the c.59926+1 G>A *TTN*tv in atrial iPSC-CMs but extends its reach to multi-scale simulations and co-culture environments, providing a nuanced understanding of the pathogenic mechanisms underlying *TTN*tv associated AF. These findings contribute to our comprehension of the disease and opens up possibilities for pharmacological intervention in patients with *TTN*tv associated structural remodelling/cardiac fibrosis.

4.5 Supplementary Figures and Tables

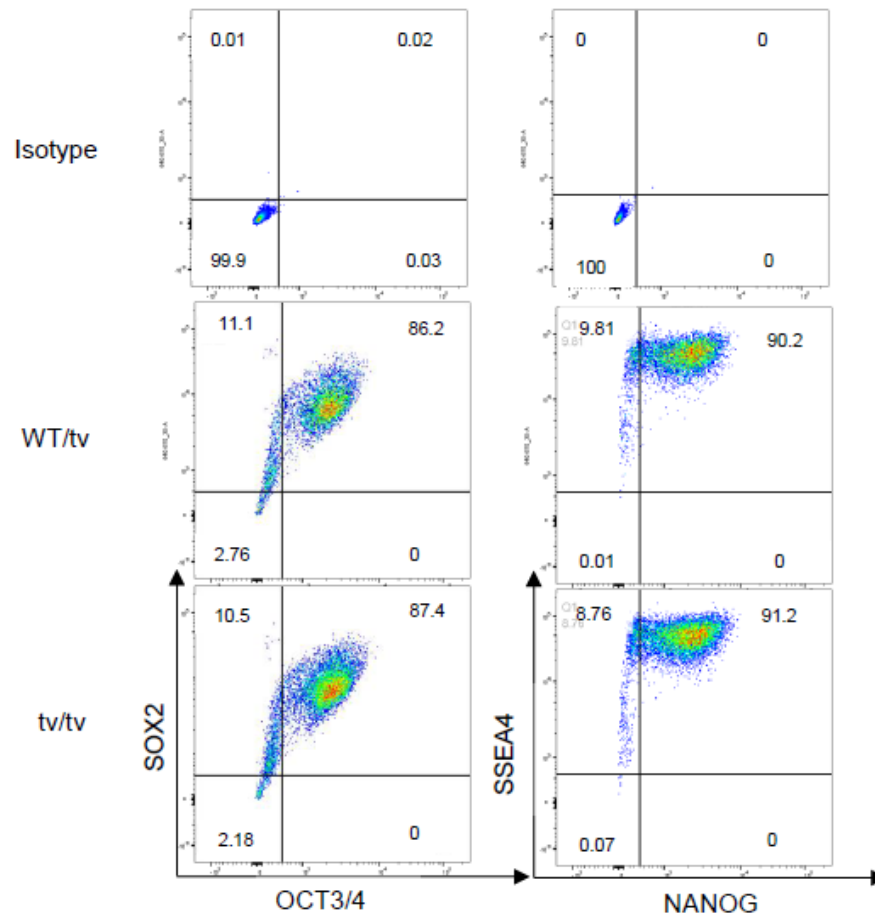


Figure 4.S1: Analysis of Protein Expression for Pluripotency Markers

Flow cytometry assays for expression of pluripotency markers SOX2, OCT3/4, SSEA4 and NANOG for both the established TTN c.59926+1^{WT/tv} and c.59926+1^{tv/tv} iPSC lines. Both were qualified as pluripotent cells due to the high expression of all the stem cell markers assessed.

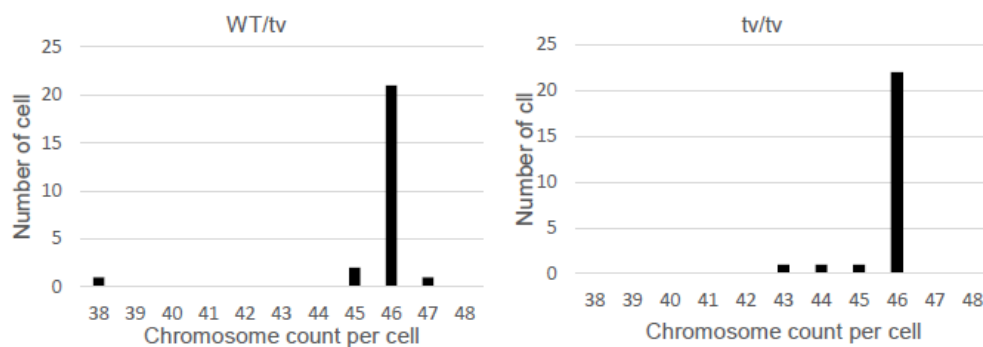


Figure 4.S2: Karyotype Analysis of TTNtv iPSC line

Distribution of chromosome count per cell at the metaphase stage for the established TTN c.59926+1^{WT/tv} and c.59926+1^{tv/tv} iPSC lines. Both were qualified as karyotypically normal due to the clear modal chromosomal number at 46.

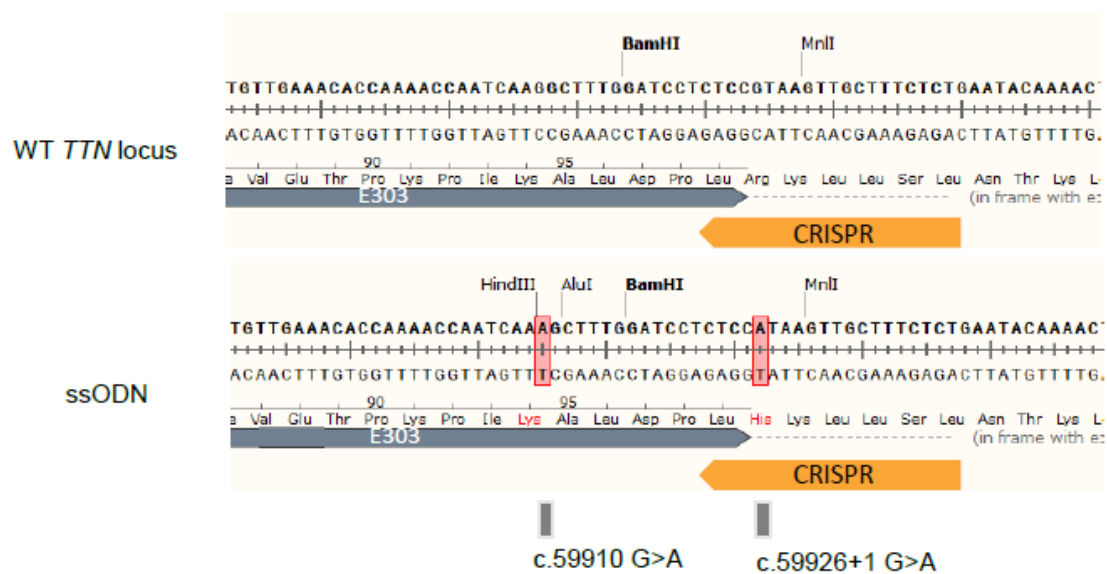


Figure 4.S3: CRISPR/Cas9 targeting strategy to incorporate TTN c.59926+1 G>A

A guide RNA containing crRNA targeting c.59926+1 G>A (5'-AGAGAAAGCAACUUACGGAG-3') was complexed with Cas9 protein and electroporated into iPSCs with a HDR template (showed here as single-stranded oligodeoxynucleotide (ssODN)) harbouring both TTNtv (TTN c.59926+1 G>A) and HindIII sites (c.59910 G>A) to generate TTNtv lines. The silent mutation corresponding to a HindIII site was modified for genotypic screening purposes.

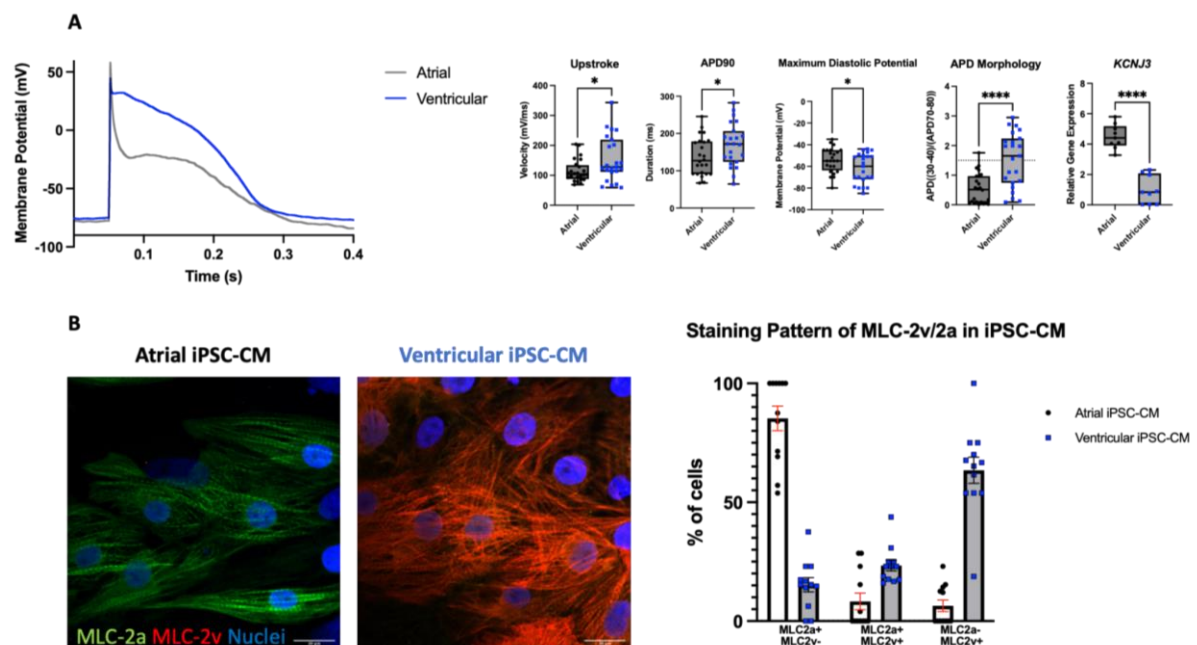


Figure 4.S4: Characterisation of Atrial and Ventricular iPSC-CMs

iPSCs were differentiated into atrial and ventricular iPSC-CM and matured until day 30. (A) Cells were dissociated, and current clamp was performed, with action potentials stimulated with a 1 nA, 2 ms depolarising current (1 Hz). Upstroke, APD90, Maximum Diastolic Potential and APD Morphology are shown above (Atrial N = 1, n = 23) (Ventricular N = 1, n = 23). A Welch's t test was used to ascertain significance (* denotes $P < 0.05$, **** denotes $P < 0.0001$) RT-qPCR was performed on the atrial specific gene marker *KCNJ3*. (B) Cells were fixed and stained for the atrial and ventricular specific isoforms of Myosin Light Chain (MLC-2a/MLC-2v). Images were taken and analysed for the proportion of cells positive and negative for the markers (N = 1, n = 12 images).

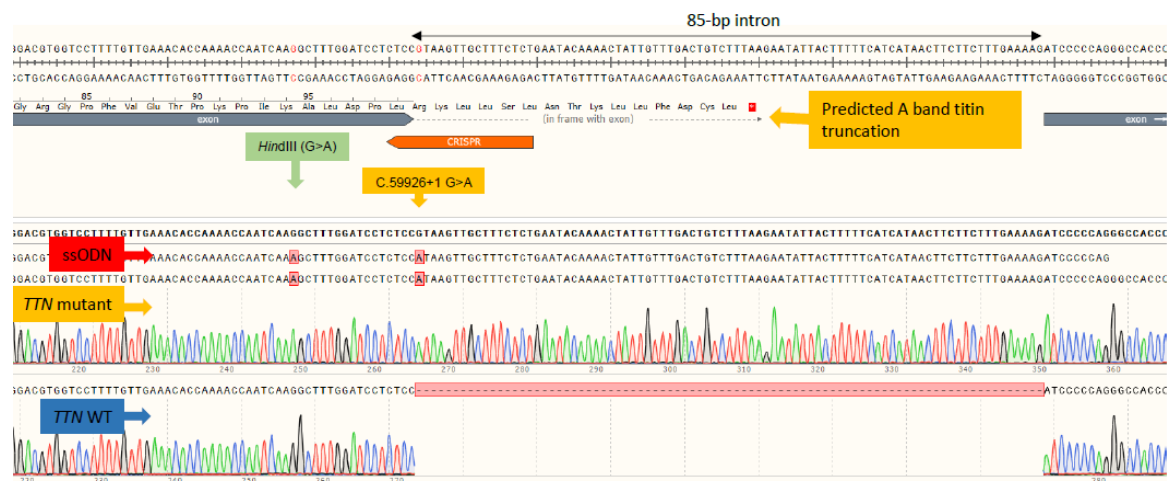


Figure 4.S5: Retention of Downstream Intron in TTN c.59926+1 G>A

DNA sequencing of the mutant *TTN* cDNA derived from the transcript carrying *TTN* c.59926+1 G>A showed additional nucleotides (85 bp) corresponding to the intron immediately downstream of exon 303 which was absent in the WT transcript. The intronic retention introduced a frameshift and premature stop codon (*TTN* p.H10911RfsX16) which may result in the generation of a truncated protein.

Table 4.S1: Taqman Probes

Target Gene	Assay ID
<i>COL1A1</i>	Hs00164004_m1 (FAM)
<i>COL3A1</i>	Hs00943786_g1 (FAM)
<i>GAPDH</i>	Endogenous Control (VIC, 4326317E)

Table 4.S2: Primers

Target Gene	Primer Sequences
<i>TTN</i> (Exon 6-7)	F: GTCCCCATCGCCCATAGAC R: GAGGCACTTCAGGACCTGTG
<i>TTN</i> (Exon 11-12)	F: CCCGGGAGCTCAAGAAGAAA R: TGACTTTGGGTGTGGCAACT
<i>TTN</i> (Exon 49-50)	F: TGAGGCCTTGAATGACAGCG R: GATCACTGGGGCAGCTCTTT
<i>TTN</i> (Exon 296-297)	F: TGCTACTGTCCAGGGTCTCA R: CTTCCAGGTCTTCAGGACGC
<i>TTN</i> (Exon 303-304) (Non qPCR)	F: AACCTGGGCCACCTAGAGAT R: GGAGACCAAGACCCACAATG
<i>TTN</i> (Exon 317-318)	F: ACCCGCTTTGAGGTTACTGG R: CCGCATCAAGTTCTCCCTCA
<i>TTN</i> (Exon 360-361)	F: GGATCTGTGTCCTCTAGCTGC R: GAAGCTTCCTCTTTGAGGGCT
<i>GAPDH</i> (Non qPCR)	F: TGCMTCTGCACCACCAACT R: YGCCTGCTTCACCACCTTC
<i>PDGFA</i>	F: TAGGGAGTGAGGATTCTTTG R: CGAGGAATCTCGTAAATGAC
<i>PDGFB</i>	F: GGGCAGGGTTATTTAATATGG R: AATCAGGCATCGAGACAG

<i>TGF-β1</i>	F: AACCCACAACGAAATCTATG R: CTTTAACTTGAGCCTCAGC
<i>KCNJ3</i>	F: GAAACAACCTGGGATGACTTG R: CCCTCTTCTAAGGAAATTACAG
<i>KCNK1</i>	F: ACTTCACCTCCGCGCTCTTCTT R: AACAGGAGGGTGAAGGGAATGC
<i>KCND2</i>	F: GCTACAGTTATGTTCTACGC R: ATACCCTAGTGTGTCATGG
<i>GAPDH</i> (qPCR)	F: TCGGAGTCAACGGATTG R: CAACAATATCCAACCTTACCAGAG

Table 4.S3: ELISA Kits

Name, Target	Company, Catalog	Type
Human TGF-beta 1 ELISA Kit, TGF-β1	RayBiotech, ELH-TGFb1-1	Sandwich
Human Pro-Collagen I alpha 1 DuoSet ELISA, Pro-Collagen 1	R&D Systems, DY6220-05	Sandwich
Human Fibronectin ELISA Kit, Fibronectin	RayBiotech, ELH-FN1-1	Sandwich

Table 4.S4: Primary and Secondary Antibodies

Target	Company, Catalog	Host	Concentration
MLC-2A (Primary)	Synaptic Systems (311 011)	Mouse	1:500
MLC-2V (Primary)	Proteintech, 10906-1-AP	Rabbit	1:500
Phalloidin (F-Actin) (Conjugated)	Thermo Fisher Scientific, A22284	N/A	1:500
α-actinin (Primary)	Abcam, ab68167	Rabbit	1: 500
Titin (T12) (Z-Disc Binding) (Primary)	Fürst et al., 1988	Mouse	2 µg/mL
Vimentin (Primary)	Abcam, ab45939	Rabbit	1:500
Alexa Fluor™ 488 anti-Mouse IgG (Secondary)	Thermo Fisher Scientific, A28175	Goat	1:500
Alexa Fluor™ 546 anti-Rabbit IgG (Secondary)	Thermo Fisher Scientific, A-11035	Goat	1:500
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Nuclear Stain) (Conjugated)	Thermo Fisher Scientific, D1306	N/A	0.1 µg/mL

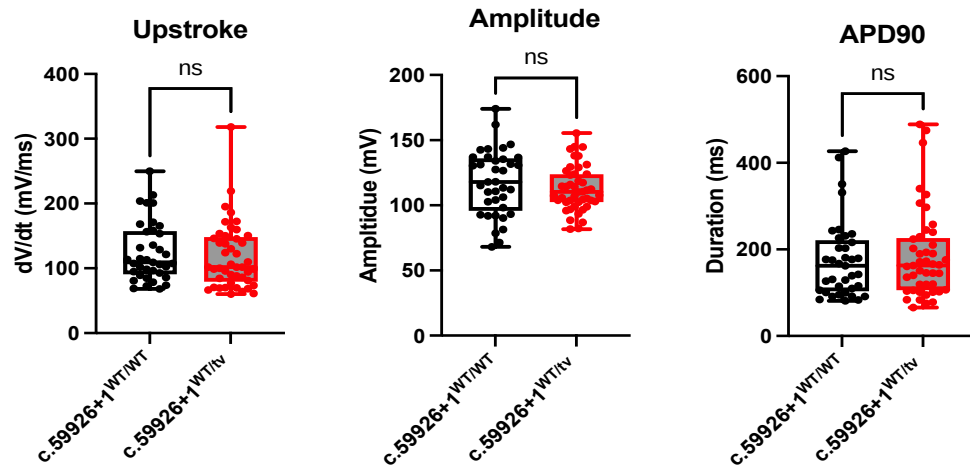


Figure 4.S6: Stimulated Action Potentials of c.59926+1^{WT/WT} and c.59926+1^{WT/tv} Atrial iPSC-CMs

Atrial iPSC-CMs were differentiated and matured until day 30. Current clamp was performed on the cells, with action potentials stimulated at 1 Hz using a 1 nA (2 ms) depolarising current. Upstroke, Amplitude and Action Potential Duration at 90 % repolarisation (APD90) are shown above. c.59926+1^{WT/WT} N = 3, n = 37 and c.59926+1^{WT/tv} N = 3, n = 45. A Welch's t-test was used to ascertain significance (ns denotes P > 0.05).

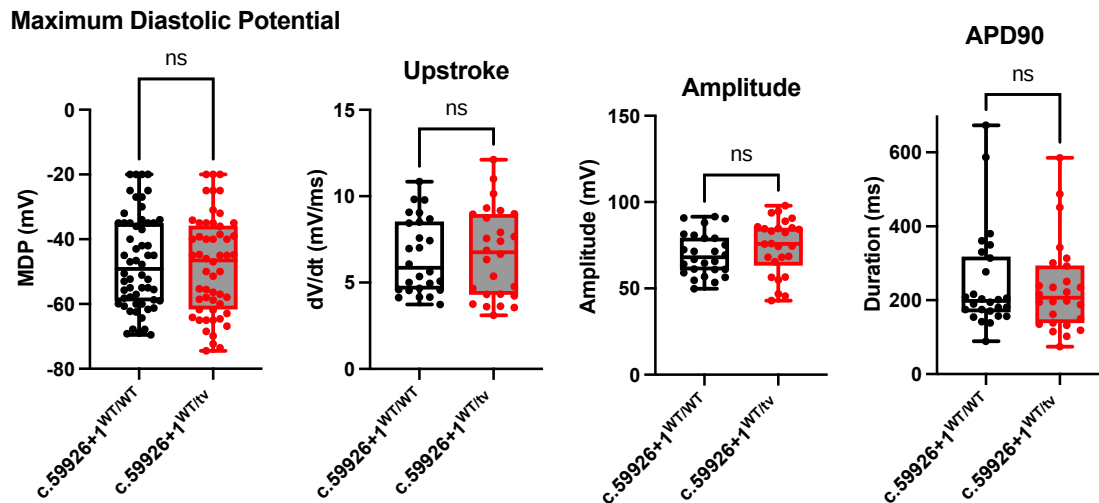


Figure 4.S7: Spontaneous Action Potentials of c.59926+1^{WT/WT} and c.59926+1^{WT/tv} Atrial iPSC-CMs

Atrial iPSC-CMs were differentiated and matured until day 30. Current clamp was performed on the cells, with spontaneous action potentials recorded over a 10 second period. Maximum Diastolic Potential (MDP), Upstroke, Amplitude and Action Potential Duration at 90 % repolarisation (APD90) are shown above. Maximum Diastolic Potential c.59926+1^{WT/WT} N = 3, n = 58 and c.59926+1^{WT/tv} N = 3, n = 54. Upstroke, Amplitude and APD90: c.59926+1^{WT/WT} N = 3, n = 26 and c.59926+1^{WT/tv} N = 3, n = 26. A Welch's t-test was used to test significance (ns denotes P > 0.05).

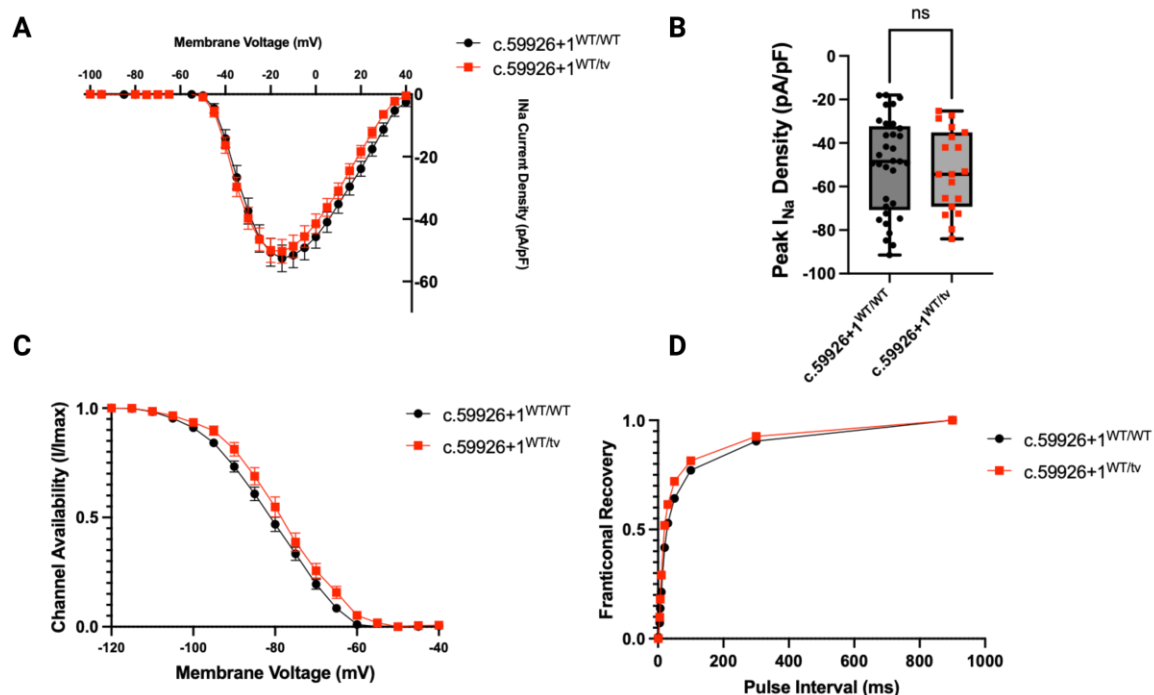


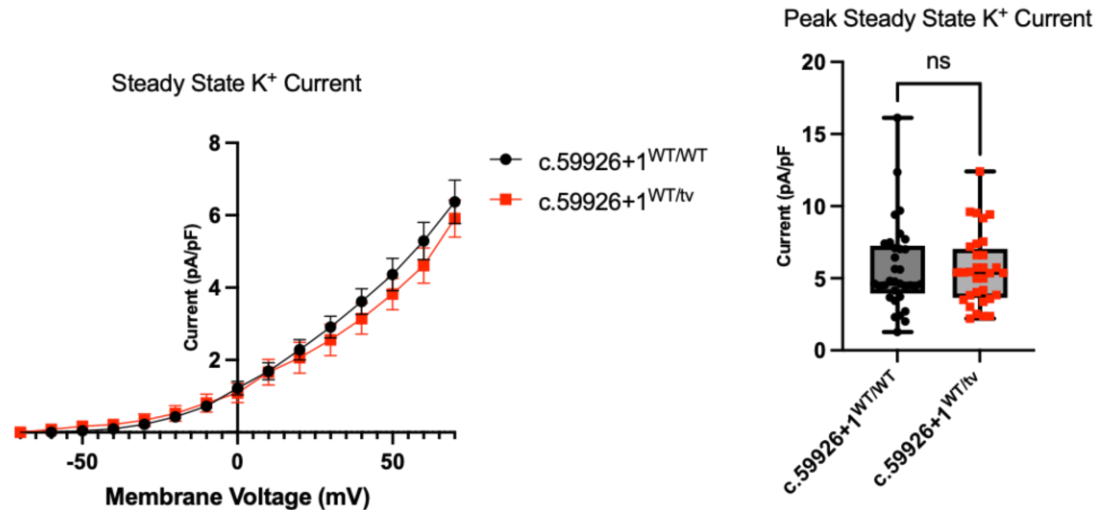
Figure 4.S8: Inward Na^+ Current of $c.59926+1^{WT/WT}$ and $c.59926+1^{WT/tv}$ Atrial iPSC-CMs

Atrial iPSC-CMs were differentiated and matured until day 30. Voltage clamp was performed for inward sodium current amplitude and kinetics. **A:** Current density – voltage (I-V) graph of the Na_v channels, **B:** Peak I_{Na} Density, **C:** Time-dependent recovery kinetics of I_{Na} , **D:** Voltage dependence of inactivation. $c.59926+1^{WT/WT}$ N = 3, n = 33 and $c.59926+1^{WT/tv}$ N = 3, n = 19. A Welch's t-test was used to test significance (ns denotes $P > 0.05$).

Table 4.S5 Biophysical Parameters of Inward Na^+ Current in $c.59926+1^{WT/WT}$ and $c.59926+1^{WT/tv}$ Atrial iPSC-CMs

Biophysical Parameter	WT	Het	Significance
Inact V50	-81.453438	-78.723333	ns
Inact K	6.819	6.934	ns
Act V50	-30.982188	-28.658421	ns
Act K	5.73175	6.23226316	ns
Recovery P50	28.3827586	20.4978889	Yes 0.0049

A



B

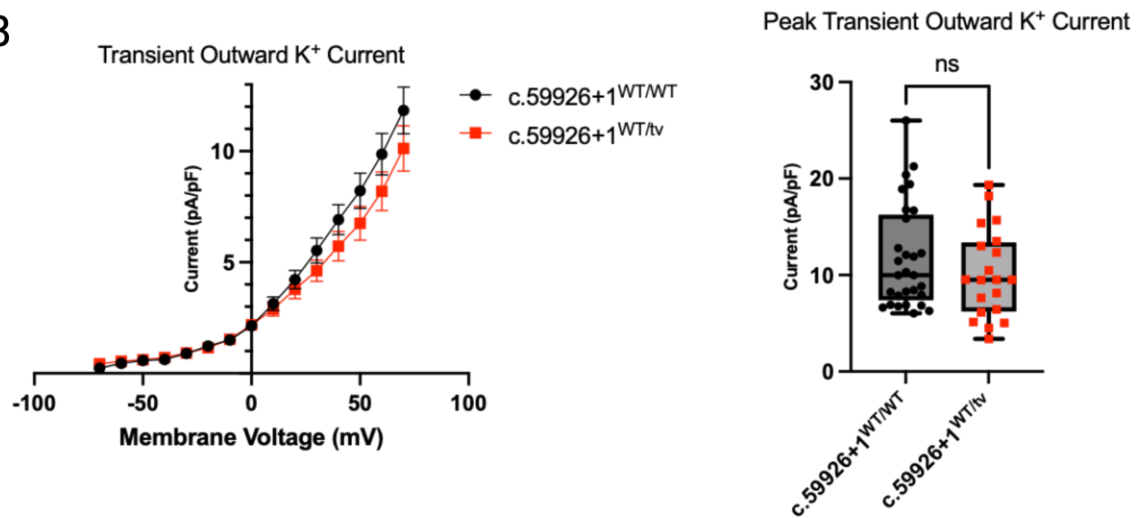


Figure 4.S9: Outward K⁺ Current of c.59926+1^{WT/WT} and c.59926+1^{WT/tv} Atrial iPSC-CMs

Atrial iPSC-CMs were differentiated and matured until day 30. Voltage clamp was performed for peak steady state and transient outward K⁺ current. **A:** Current density – voltage (I-V) graph and peak steady state K⁺ current, **B:** Current density – voltage (I-V) graph and peak transient outward K⁺ current. Peak transient outward K⁺ current c.59926+1^{WT/WT} N = 3, n = 29 and c.59926+1^{WT/tv} N = 3, n = 20. Peak steady state K⁺ current c.59926+1^{WT/WT} N = 3, n = 33 and c.59926+1^{WT/tv} N = 3, n = 32. A Welch's t-test was used to test significance (ns denotes P > 0.05).

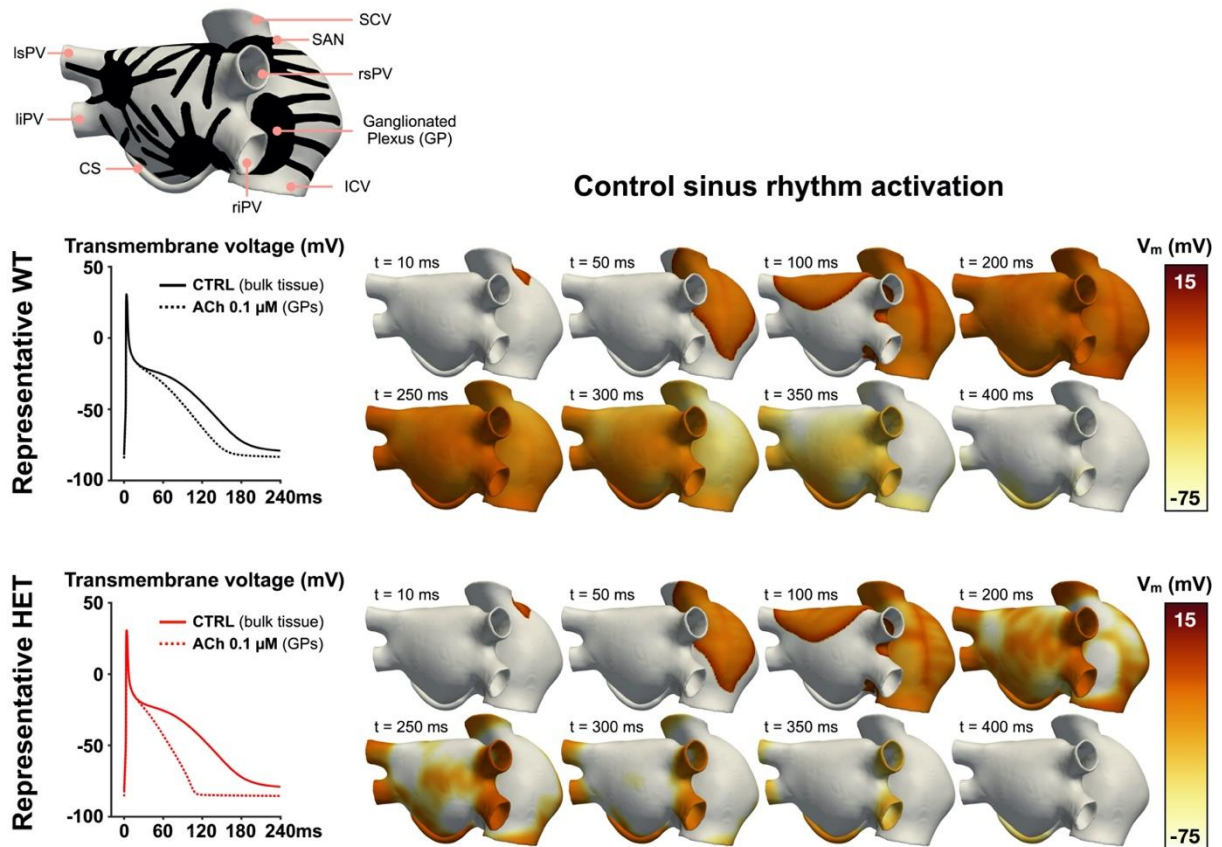


Figure 4.S10: Heterogeneous repolarisation for heterozygous (HET) conditions during control sinus rhythm activation

Top: Schematic representation of the human whole-atria model used for multi-scale simulations. Abbreviations. SAN: sinoatrial node; SCV-ICV: superior and inferior cava vein; rs-ri-ls-li-PV: right superior, right inferior, left superior and left inferior pulmonary vein; CS: coronary sinus. The release of 0.1 μ M acetylcholine only occurs at the ganglionated plexuses (GPs), arranged following an octopus configuration. **Bottom:** Atrial cardiomyocyte models used for wild type (WT) c.59926+1^{WT/WT} and heterozygous (HET) c.59926+1^{WT/tv} conditions. (left). Consecutive snapshots of the atrial transmembrane voltage (V_m) during control sinus rhythm activation. The considerably shorter APD in GPs sites for HET conditions creates a heterogeneous repolarisation (left).

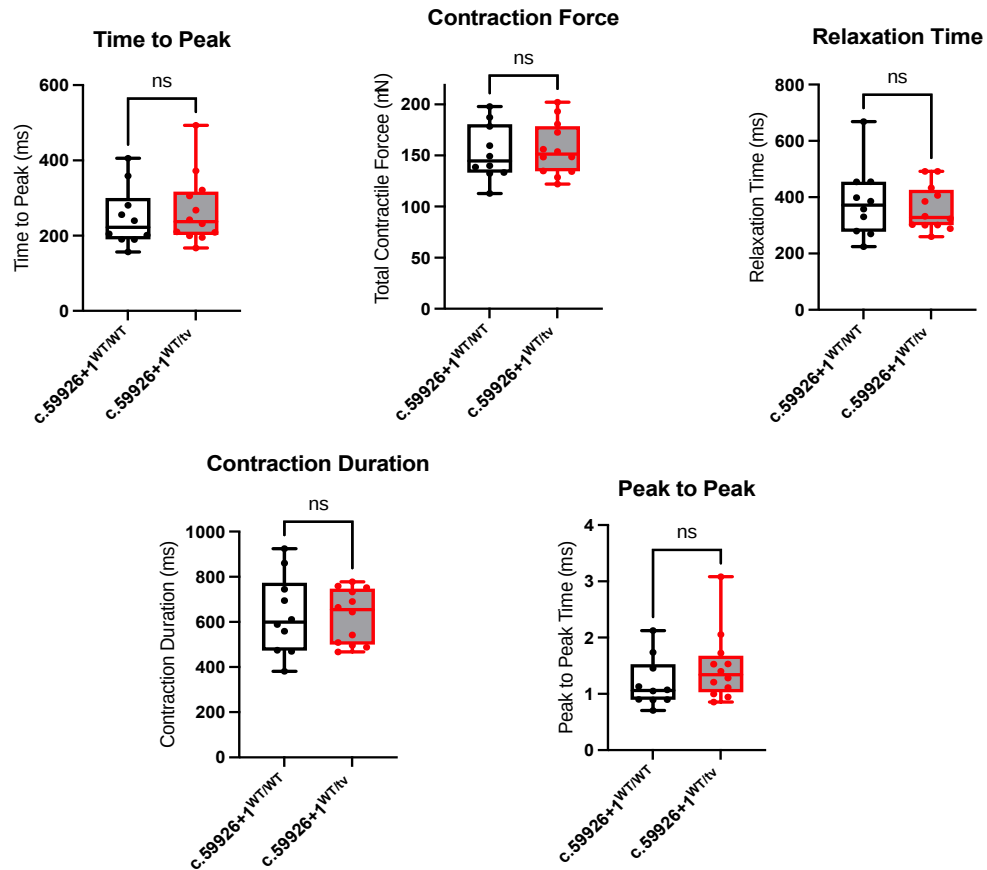


Figure 4.S11: Contractile Analysis of *c.59926+1^{WT/WT}* and *c.59926+1^{WT/tv}* Engineered Heart Tissues

Videos of day 30 EHTs were recorded and turned into tif stacks. An in-house software was used to track the movement of the pillars during contraction. Contraction duration, Peak to Peak time, Time to Peak time, Relaxation Time and Contraction Force are shown above. *c.59926+1^{WT/WT}* N = 3, n = 10 EHTs and *c.59926+1^{WT/tv}* N = 3, n = 12 EHTs. A Welch's t-test was used to test significance (ns denotes $P > 0.05$).

Chapter 5: Investigating the physiological relevance and utility of induced pluripotent stem cell derived cardiac fibroblasts

Parts of the Materials and Methods and Results of this Chapter are taken from previously submitted work: Cumberland, M.J., Euchner, J., Azad, A.J., TN Vo, N., Kirchhof, P., Holmes, A.P., Denning, C. and Gehmlich, K., 2023. Generation of a human iPSC-derived cardiomyocyte/fibroblast engineered heart tissue model. *F1000Research*, 12, p.1224. Mass Spectrometry runs were performed and delineated by Dr Jinglei Yu in 5.2.4.3.

5.1 Introduction

5.1.1 Cardiac Fibrosis

Cardiac fibrosis describes the process whereby cardiac fibroblasts become activated and an accumulation of extracellular matrix takes place in and around cardiac muscle (Frangogiannis, 2021). It often causes the stiffening of the heart and impedes cardiac function. Although present in nearly all types of chronic heart disease, cardiac fibrosis is not always malign and can be seen as a maladaptive response to cardiac stress. For example, following myocardial infarction and the death of cardiac myocytes, the heart is unable to regenerate and consequently forms scar tissue composed predominantly of collagen to maintain structural integrity and avoid rupture. Although beneficial in the short term, the formation of the fibrotic scar tissue can ultimately cause cardiac dysfunction and an increased proclivity for arrhythmia (Yin et al., 2023). There are three main types of cardiac or myocardial fibrosis: replacement fibrosis, reactive interstitial fibrosis and infiltrative interstitial fibrosis (Hinderer and Schenke-Layland, 2019). Replacement fibrosis describes the aforementioned replacement of necrotic cardiomyocytes and is broadly seen as reparative. Interstitial fibrosis occurs in the absence of severe cardiomyocyte loss and is observed following changes in myocardial load (reactive) or glycolipid build-up (infiltrative).

5.1.2 Cardiac Fibrosis and Heart Disease

Cardiac fibrosis can be both a consequence or cause of various heart diseases including arrhythmia, cardiomyopathy and heart failure (Sohns and Marrouche, 2020) (Segura et al., 2014) (Eijgenraam et al., 2020). In AF, atrial fibrosis can act as a substrate for arrhythmia by disrupting signal propagation and promoting re-entrant circuits (Burstein and Nattel, 2008). Cardiac fibrosis can manifest early in the development of hypertrophic and dilated cardiomyopathy, decreasing cardiac performance and acting as risk factor for arrhythmia

(Cojan-Minzat et al., 2021) (Schlittler et al., 2023). Furthermore, cardiac fibrosis can promote cardiac hypertrophy, chamber dilatation and expedite diastolic and systolic dysfunction in heart failure (Travers et al., 2016). For this reason, anti-fibrotics have been seen as a viable and promising strategy for the pharmacological treatment of cardiovascular disease (Fang et al., 2017). Identifying anti-fibrotics that have universal effect is however difficult, as the fibrotic response in chronic heart disease is often heterogeneous and propagated by diverse aetiologies. Furthermore, large phenotypic variation between subpopulations of cardiac fibroblasts makes elucidating and reversing the mechanisms behind cardiac fibrosis difficult (Díez and de Boer, 2022).

5.1.3 Cardiac Fibroblasts

Cardiac fibroblasts are one of the most populous non-myocyte cell types present in the heart and provide structure and support to the cardiac chambers (Ivey and Tallquist, 2016). The proportion of cardiac fibroblasts present can depend on age, chamber and disease state of the heart, with a higher proportion of cardiac fibroblasts having been reported in the ventricles of failing hearts (Tucker et al., 2020) (Banerjee et al., 2007) (Wang et al., 2020) (Nagaraju et al., 2019) (Sridhar et al., 2017). Defining what a cardiac fibroblast is can be difficult, due to the subpopulations present and the lack of uniquely expressed markers (Doppler et al., 2017). Cardiac fibroblasts are flat, spindle shaped cells that lack a basement membrane (Souders et al., 2009). The cytoskeletal and extracellular proteins vimentin and collagen type 1 have proven useful markers and can be combined with others including discoidin domain receptor 2 (DDR2) and fibroblast specific protein 1 (FSP1) to accurately identify cardiac fibroblasts (Snider et al., 2009). Cardiac fibroblasts are interspersed between heart muscle fibres. Their primary function is to act as a scaffold to maintain the structural integrity of the heart and to orchestrate the organisation of the extracellular matrix (Eghbali et al., 1988). In addition to

this, cardiac fibroblasts can produce paracrine signalling molecules that can alter cardiomyocyte contraction and proliferation, apoptosis, and vessel homeostasis (Krenning et al., 2010). Cardiac fibroblasts can directly and indirectly influence the electrophysiology of the heart. Direct coupling of cardiac fibroblasts with cardiac myocytes through connexins has been demonstrated *in vitro* and can cause changes to the membrane potential of the myocyte, whilst deposition of extracellular matrix can impede signal propagation and cardiac conduction (Vasquez et al., 2011) (Schultz et al., 2019). Many of the normally beneficial functions of cardiac fibroblasts are perturbed during the pathological remodelling of the heart.

5.1.4 Cardiac Fibroblast Activation and Myofibroblasts

The primary driver of cardiac fibrosis is the activation of cardiac fibroblasts into myofibroblasts caused by acute or chronic injury. The myofibroblast has a greater propensity for ECM secretion and can produce contractile force due to the expression of alpha smooth muscle actin (α SMA) microfilaments (stress fibres) (Aujla and Kassiri, 2021). Following activation, myofibroblasts converge to the region of injury to facilitate extracellular matrix deposition and the release of pro-fibrotic factors (Kong et al., 2014). The release of growth factors and cytokines, including $\text{TNF}\alpha$, IL6 and $\text{TGF}\beta$, further exacerbates the response and activation of cardiac fibroblasts (Ma et al., 2018). Cardiac fibroblast activation is a complex process which can be stimulated by a variety of mechanical and chemical stimuli. Such stimuli include: angiotensin II, aldosterone, β -adrenergic catecholamines, inflammatory mediators (e.g. $\text{TGF}\beta$), platelet derived growth factors, Wnt signalling, endothelin-1, ECM proteins, syndecans, oxidative stress and non-coding RNAs (Frangogiannis, 2019). The stimuli relevant to this thesis are discussed in more detail below.

5.1.4.1 *TGF- β Mediated Cardiac Fibroblast Activation*

Transforming growth factor β (TGF- β) is the most researched fibrogenic growth factor and often plays a determining role in cardiac fibrosis. TGF- β 1 is the predominantly expressed isoform in the heart and promotes cardiac fibroblast activation through Smad-dependent or independent pathways. In the Smad-dependent pathway, TGF- β 1 binds to receptors on the cell surface membrane, which phosphorylate and activate the intracellular transcription factors Smad2 and Smad3 (Zhang et al., 2022). Activated Smad2 and Smad3 can cause the upregulation of pro-fibrotic and ECM genes (*COL1A1*, *COL1A2*, *COL3A1*, *TNF*, *FN1*) and the assembly of α SMA stress fibres (Huang et al., 2020) (Hanna et al., 2021). The Smad-independent pathway involves a signalling cascade triggered by TGF- β mediated activation of mitogen activated protein kinases (MAPKs) such as TAK1. This leads to the activation of p38 and JNK, which can promote cardiac fibroblast differentiation and interstitial fibrosis (Aujla and Kassiri, 2021).

5.1.4.2 *Renin-Angiotensin-Aldosterone Mediated Cardiac Fibroblast Activation*

The renin-angiotensin-aldosterone system is activated during pathological remodelling of the heart and plays a crucial role in the differentiation of cardiac fibroblasts to myofibroblasts (Weber et al., 2013). Stressed cardiac fibroblasts and cardiomyocytes synthesize angiotensin converting enzyme (ACE) and renin to help facilitate production of the pro-fibrotic and cardiac fibroblast activating hormone angiotensin II (Sadoshima et al., 1993). Angiotensin II binds to type I angiotensin receptors on cardiac fibroblasts and promotes activation, proliferation and increased ECM deposition (Frangogiannis, 2019). This activation is mediated through signalling cascades mediated by mitogen activated protein kinase, protein kinase C and extracellular signal-regulated kinase (ERK) (Sano et al., 2001). Binding of angiotensin II to angiotensin type II receptors causes an antagonistic effect on proliferation

and ECM synthesis. Pro-inflammatory cytokines including TGF- β 1 help modulate the responsiveness of cardiac fibroblasts to angiotensin II mediated activation. For example, IL-1 β and TNF α can promote the expression of angiotensin type I receptors in cardiac fibroblasts, increasing the pro-fibrotic effect of angiotensin II (Gurantz et al., 2005). Increased production of angiotensin II conversely inhibits transcription of the angiotensin type I receptor gene *AGTR1* (Everett et al., 1996). Generating a negative feedback loop that prevents uncontrolled fibrogenesis. As well as its direct pro-fibrotic effect on cardiac fibroblasts, angiotensin II can stimulate the secretion of the hormone aldosterone in both the adrenal cortex and the heart (Buffolo et al., 2022). Aldosterone can activate mitogen activated protein kinase cascades in cardiac fibroblasts to promote proliferation, migration, and extracellular matrix synthesis (Stockand et al., 2003).

5.1.4.3 β -Adrenergic Activation of Cardiac Fibroblasts

Adrenergic activation of cardiac fibroblasts by catecholamines including adrenaline and noradrenaline can induce myocardial fibrosis and pathological cardiac remodelling (Benjamin et al., 1989) (Werhahn et al., 2021). Adrenergic stimulation can contribute towards myocardial fibrosis through both indirect and direct mechanisms. Indirectly, chronic adrenergic stimulation of the heart can cause the release of pro-fibrotic factors from stimulated cardiomyocytes, cause cardiomyocyte necrosis and concomitant reparative fibrosis, and stimulate the activation of macrophages (Noh et al., 2017) (Frangogiannis, 2019) (Benjamin et al., 1989). Directly, activation of the β -Adrenergic receptors of cardiac fibroblasts can stimulate proliferation through phosphatidylinositol 3-kinase and mitogen activated protein kinases (Colombo et al., 2001) (Molkentin et al., 2017). There are 3 types of G-protein coupled β -adrenergic receptors in the heart: β 1, β 2, and β 3 (Ali et al., 2020). The binding of agonists including adrenaline and noradrenaline to adrenergic receptors ultimately leads to the activation of G protein-coupled receptor kinase 2 (GRK2) (Travers et al., 2017).

The molecular mechanisms behind GRK2 mediated cardiac fibroblast activation remain unknown. Similarly, the exact role each type of receptor plays in regulating cardiac fibrosis in the heart is still unclear. Sympathetic activation of $\beta 1$ receptors is broadly thought to promote cardiac fibroblast proliferation and adverse remodelling. Conversely, the impact $\beta 2$ activation has on cardiac fibrosis is more complex, with studies demonstrating both pro and anti-fibrotic effect (Imaeda et al., 2019) (Zhu et al., 2020).

5.1.4.4 Platelet Derived Growth Factors (PDGFs)

Platelet derived growth factors (PDGFs) (PDGF-AA, BB, AB, CC, DD) are a group of homo or heterodimeric heparin binding growth factors capable of binding to and acting on PDGFR α and PDGFR- β receptors. PDGFs are integral to heart development and are capable of interacting with a broad range of mesoderm-derived cells, including cardiac fibroblasts (Raica and Cimpan, 2010). An extensive number of *in vitro* and *in vivo* studies have demonstrated that PDGF isoforms play a crucial role in the development of cardiac fibrosis (Raines, 2004). Activation and overexpression of PDGF receptors and ligands is often induced by mechanical stress and is associated with the development of atrial fibrosis, atrial fibrillation and arrhythmogenic cardiomyopathies (Ma et al., 1999) (Lee et al., 2019) (Chen et al., 2022) (Jiang et al., 2016). PDGF-A and PDGF-D are potent pro-fibrotics that can bind to the PDGFR- α and - β receptors of cardiac fibroblasts and promote proliferation and ECM deposition (Simm et al., 1998) (Zhao et al., 2013). Following binding, the PDGF receptor dimerises and activates to initiate intracellular signalling cascades including the MAPK, PI3K/Akt and JAK/STAT pathways (Fan et al., 2014). Activation of these pathways leads to transcriptional changes in cardiac fibroblasts that promote ECM expression, cell proliferation and consequently myocardial fibrosis.

5.1.4.5 Endothelin-1

Endothelin-1 (ET-1) is a pro-fibrotic peptide predominantly produced by vascular endothelial cells, as well as cardiomyocytes, cardiac fibroblasts, and smooth muscle cells (Rodríguez-Pascual et al., 2011). ET-1 has been implicated as a potent pro-fibrotic in the development of cardiac fibrosis and has previously been evaluated as a potential prognostic and predictive marker for heart failure and myocardial infarction (Jankowich and Choudhary, 2020). It is currently unclear as to what extent the fibrogenic effects of ET-1 are caused by direct cardiac fibroblast activation. Previous studies have demonstrated that ET-1 can bind to endothelin receptors (ET_A and ET_B) on cardiac fibroblasts and induce myofibroblast differentiation through activation of the MAPK and ERK intracellular signalling pathways (Shi-Wen et al. 2004). However, the pro-fibrotic effects of ET-1 are strongly associated with those of TGFβ. ET-1 potentiates TGFβ dependent expression of pro-fibrotic genes and proteins through the upregulation of TGFβ receptor genes (Wermuth et al., 2016). Whilst *in vitro* studies have demonstrated that TGFβ stimulates increases expression of ET-1 through JNK signalling and that ET-1 is integral to its pro-fibrotic function (Shi-wen et al., 2007). Similarly, other studies have suggested that ET-1 is a mediator of fibrosis and works downstream of other pro-fibrotics including angiotensin II (Leask, 2010).

1.6.3.2 Electrophysiological Activation of Cardiac Fibroblasts

Expression and function of mechanosensitive and voltage gated ion channels is thought to be important in the activation of cardiac fibroblasts (Stewart and Turner, 2021) (Xing et al., 2023). However, the role ion currents have on the activation and subsequent electrophysiology of myofibroblasts is not fully understood and likely requires further research (Xing et al., 2023). Cardiac fibroblasts are not considered an electrically excitable cell type. Nevertheless, the electrophysiological properties of cardiac fibroblast have emerged as a pertinent topic of research in cardiac fibrosis for two principal reasons. Firstly, ion

channels are important to cardiac fibroblast activation. During myofibroblast differentiation, there is an influx of Ca^{2+} into the cell which can activate calcineurin and cause NFAT mediated expression of pro-fibrotic genes including *ACTA2* (αSMA) and *COL3A1* (Collagen 3) (Herum et al., 2013). This influx of Ca^{2+} into the cell is broadly thought to be due to the activity of mechanosensitive cation non-specific piezo and transient receptor protein (TRP) ion channels (Stewart and Turner, 2021). The transient receptor protein (TRP) channels are a group of non-selective cation channels, which can respond to mechanical stimuli within the heart. Activation of TRP channels is broadly associated with calcium influx.

Secondly, changes in ionic current during cardiac fibroblast activation can have deleterious electrophysiological ramifications in the heart. Following activation of cardiac fibroblasts into myofibroblasts, the membrane potential of the cells becomes even further depolarised due to the increase in intracellular Ca^{2+} as well as putative changes in the activity of K^+ channels including: $\text{K}_v2.1$, $\text{K}_{ir}2.1$ and large conductance potassium channels (BK_{Ca} channels) (Feng et al., 2019) (Poulet et al., 2016) (Qi et al., 2015). As previously mentioned, heterocellular coupling can occur between cardiomyocytes and cardiac fibroblasts.

Depolarisation of the myofibroblast membrane potential is thought to cause an increase in adjacent myocytes resting membrane potential. This can cause arrhythmogenic changes including slowing of cardiac conduction (reduced availability of Na^+ channels) and increased ectopic activity (Miragoli et al., 2006) (Zlochiver et al., 2008).

The role ion currents have on cardiac fibroblast activation and phenotype is not fully understood. Significant heterogeneity has been observed in the currents recorded from cardiac fibroblast cell types, with much of this variation occurring due to differences in species, time in culture, disease state and tissue compartment (Vasquez et al., 2011).

Research into changes that occur in cardiac fibroblast electrophysiology during fibrosis have been hampered by practical complications. Accessibility of freshly isolated human cardiac

fibroblasts is limited, and although readily expanded in culture, cardiac fibroblasts can become easily activated by stiff tissue culture plastics or transdifferentiate over time (Gilles et al., 2020). Induced pluripotent stem cell derived cardiac fibroblasts (iPSC-CFs) overcome some of these limitations and have emerged as novel and practical model to explore the pathophysiology of cardiac fibrosis.

5.1.5 Induced Pluripotent Stem Cell derived Cardiac Fibroblasts

There is an inexorable shift towards the replacement of animal models with more physiologically relevant human alternatives. iPSC-CFs offer a human alternative to animal cardiac fibroblasts and are capable of circumventing some of the limitations associated with acquiring and maintaining primary human cardiac fibroblasts. iPSC-CFs can be differentiated and maintained under controlled quiescent conditions, mitigating unwanted activation and transdifferentiation of the cells. Furthermore, they show greater plasticity for activation than their primary counterparts, making them a useful tool for anti-fibrotic drug screenings (Floy et al., 2021). As the cells are derived from iPSCs, they represent a genetically malleable source of cells in which pathogenic variants associated with cardiac fibrosis can be explored using CRISPR-Cas9 gene editing (Soussi et al., 2023). Previously developed protocols for the differentiation of iPSC-CFs generate cells that resemble primary ventricular fibroblasts at the morphological, structural, transcriptional, and functional level (H Zhang et al., 2019) (J Zhang et al., 2019). Furthermore, a study investigating the effect TGF- β 1 mediated activation has on primary ventricular and iPSC-CFs demonstrated similarities in the response of the cells (Moita et al., 2022). Limited work has been published on the differences between cardiac fibroblasts derived from the upper and lower chambers of the heart. Although demonstrating broad similarity, contrasts have been identified in morphology, proliferation rate, PDGF expression and plasticity (Burstein et al., 2008) (Rizvi et al., 2016). To our

knowledge, no previous study has compared the physiology of iPSC-CFs and primary atrial cardiac fibroblasts.

Further validation of iPSC-CFs is needed to adequately convince many researchers of the physiological relevance of this alternative modelling system. Exploring the functional changes that occur in the ion channels of iPSC-CFs will prove useful validating the model and may provide fresh insight into the recondite mechanisms underpinning cardiac fibroblast electrophysiology.

5.1.6 Hypothesis and Aims

Hypothesis:

iPSC-CFs will resemble primary cardiac fibroblasts in their transcriptional response to TGF- β 1 induced activation. Significant changes in ion channel expression will be observed following treatment with TGF- β 1 and will cause measurable differences in ionic current. Such changes will correspond with the depolarisation of the membrane potential.

Aim:

Validate that iPSC-CFs are a suitable model in which the pathophysiology of cardiac fibrosis can be explored. Identify and validate changes in ion channel expression and function that may be integral to the activation of cardiac fibroblasts.

Objectives:

1. Assay whether iPSC-CFs, ventricular cardiac fibroblasts (VCFs) and atrial cardiac fibroblasts (ACFs) can be readily activated through the addition of the pro-fibrotic cytokine TGF- β 1
2. Identify changes in ion channel gene and protein expression that occur during and after cardiac fibroblast activation
3. Validate changes in ion channel expression by measuring currents in cardiac fibroblasts using single cell voltage clamp

5.2 Materials and Methods

5.2.1 Cardiac Fibroblasts

5.2.1.1 Cardiac Fibroblast Cell Lines

Three different cardiac fibroblast cell lines were used in the project: iPSC derived cardiac fibroblasts (iPSC-CFs) (derived in house from KOLFC2 iPSCs), primary ventricular cardiac fibroblasts (VCFs) (PromoCell, C-12375) and primary atrial cardiac fibroblasts (ACFs) (Lonza, CC-2903).

5.2.1.2 Cardiac Fibroblast Cell Culture

All cardiac fibroblasts were cultured under the same conditions. Cells were cultured on 1:100 geltrex coated 6 well plates, 24 well plates, T25 or T75 flasks. Cardiac fibroblasts were cultured in Fibroblast Growth Medium 3 (Promocell, C-23130) supplemented with 10 ng/ml Recombinant Human FGF basic/FGF2/bFGF (146 aa) Protein (bio-technie, 233-FB-010) and 10 μ M of the TGF- β 1 inhibitor SB431542 (bio-technie, 1614). Cardiac fibroblasts were passaged at 80 % confluency. Cells were dissociated by washing once with PBS, and then incubating in TrypLE™ Express for 3 minutes at 37 °C. Cells can be dislodged from the surface of the flask by gently tapping the sides of the container. Following the detachment of the cells, a 2 x volume of DMEM/F12 was added to dilute the TrypLE. The TrypLE/cell mix was centrifuged at 300 g for 3 minutes. The cells were resuspended in Fibroblast Growth Medium 3 supplemented with 10 ng/mL FGF2 and 10 μ M SB431542 and seeded at a density of 10,000 cells/cm².

5.2.1.3 Differentiation of iPSC derived Cardiac Fibroblasts

The method for the differentiation of quiescent iPSC-CFs was broadly adapted from the protocol outlined in Zhang et al., (2019) and is detailed in Cumberland et al., (2023) (*Figure 5.1*). iPSCs (Kolfc2/WTSli018-B) were seeded at 30,000 cells/cm² into a T25 flask pre-coated with 1:100 geltrex for 2 hours at 37 °C, in 10 µM Rock Inhibitor (Y-27632) mTeSR Plus medium. The cells were cultured for 48 hours prior to the medium being changed with RPMI 1640 supplemented with B27 minus insulin and 6 µM CHIR-99021 (TOCRIS, 4423) for 48 hours. The medium was then changed with RPMI 1640 supplemented with B27 minus insulin for 24 hours. The cells were subsequently treated with RPMI 1640 with B27 minus insulin and 5 µM IWR1 (Sigma-Aldrich, I0161) for 48 hours. The cells were dissociated by washing with 3 mL PBS and then treating with 5 mL accutase enzyme (Sigma-Aldrich, A6964) for 3 minutes at 37°C. The cells were removed from the flask, diluted with 5 mL pre-warmed RPMI 1640 and spun down at 200 g for 3 minutes. The cells were resuspended in Advanced DMEM/F-12 (Thermo Fisher Scientific, 12634010) supplemented with 5 µM CHIR-99021 and 2 µM retinoic acid and seeded at 20,000 cells/cm² in a Geltrex coated T25 flask. The cells were incubated in this medium for 3 days and then subsequently changed to Advanced DMEM/F-12 for a further 4 days. The cells were passaged and plated into a Geltrex coated T25 flask at a density of 20,000 cells/cm² in Fibroblast Growth Medium 3 supplemented with 10 ng/mL FGF2 and 10 µM of the TGF-β1 inhibitor SB431542. The cells were cultured in this media for 6 days, with media changes taking place every 48 hours. Following the 6 days, the cells can be assessed for purity using immunocytochemistry. It is recommended that the cells are expanded, passaged at 70-80 % confluency for 2 passages and cryopreserved for use in future experiments in serum free freezing medium.

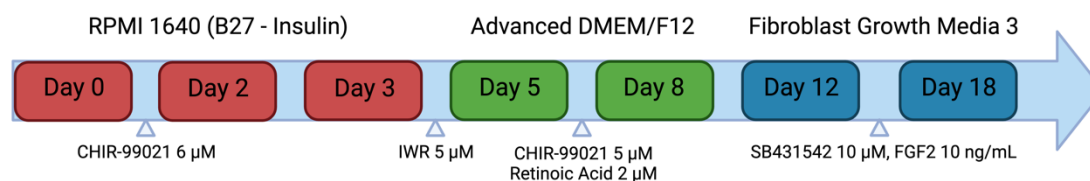


Figure 5.1: Differentiation of iPSC-CF

Schematic representation of the iPSC-CF differentiation protocol. Mesodermal specification is achieved through the activation of the Wnt signalling pathway using CHIR-99021. Subsequent inactivation of the Wnt signalling pathway by the small molecule IWR1 allows the generation of cardiac progenitor cells. Culture in CHIR-99021 and retinoic acid, drives the cells towards the derivation of cardiac fibroblasts. Cardiac fibroblasts are maintained in medium supplemented with FGF2 and the TGF- β 1 inhibitor SB431542 to prevent activation into myofibroblasts.

5.2.1.4 Immunofluorescence of Cardiac Fibroblasts

All immunofluorescence experiments were carried out on cells cultured and stained in Corning™ Costar™ 24-well plates (Fisher Scientific, 10380932). Cardiac fibroblasts were passaged into wells of a 24 well plate coated 1:100 with Geltrex at a seeding density of 10,000 cells/cm². Cells were fixed for 14 minutes at room temperature using 4 % paraformaldehyde solution (PBS) (Thermo Fisher Scientific, J19943.K2). Fixed cells were blocked for 1 hour in blocking buffer consisting of PBS, Fetal Bovine Serum (5 %), Bovine Serum Albumin (1 %) (Merck Millipore, A9418-10G) and Triton™ X-100 (0.5 %) (Merck Millipore, X100-100ML). Primary antibodies were added at dilutions listed in *Table 5.1* in blocking buffer for 2 hours at room temperature. This was followed by 3 washes in blocking buffer and the addition of secondary antibodies (*Table 5.1*) for 1 hour in the dark at room temperature. DAPI (Thermo Fisher Scientific, D1306) was added with the secondaries at 0.1 μg/mL to visualise nuclei. Samples were washed 3 times in blocking buffer and then once with PBS. All images of cardiac fibroblasts were taken using an EVOS™ M5000 Imaging System (Thermo Fisher Scientific, AMF5000).

Table 5.1: Immunofluorescence Antibodies

Target	Company, Catalog	Host	Concentration
Vimentin (Primary)	Abcam, ab45939	Rabbit	1:500
α-Smooth Muscle Actin (Primary)	Merck Millipore, A5228	Mouse	1:500
Collagen 1 (Primary)	Abcam, ab34710	Rabbit	1:500
Alexa Fluor™ 488 anti-Mouse IgG (Secondary)	Thermo Fisher Scientific, A28175	Goat	1:500
Alexa Fluor™ 546 anti-Rabbit IgG (Secondary)	Thermo Fisher Scientific, A-11035	Goat	1:500
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Nuclear Stain) (Conjugated)	Thermo Fisher Scientific, D1306	N/A	0.1 µg/mL

5.2.2 RT-qPCR of Cardiac Fibroblasts

5.2.2.1 RNA Extraction and Isolation

Previously harvested dry cell pellets, stored at -80 °C, were thawed on ice and subsequently processed. Homogenisation of samples was performed using QIAshredder (QIAGEN, 79656). RNA isolation was performed using a RNeasy Mini Kit (QIAGEN, 74104) and performed according to the protocol stipulated by the manufacturer. RNA concentration and purity was assessed using a NanoDrop 2000 (Thermo Fisher Scientific, ND-2000).

5.2.2.2 cDNA Conversion

Following quantification, 200 ng of RNA was converted into cDNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Fisher Scientific, 10400745) to give cDNA samples at 10 ng/µl. Reaction mixtures and thermal cycling conditions were prepared and set up as outlined within the product information sheet provided.

5.2.2.3 Quantitative Polymerase Chain Reaction Setup (qPCR)

Two types of qPCR setups were used. TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, 4444556) was used in combination with Taqman probes, with reactions setup in

accordance with the included protocol. 10 ng of cDNA was included within each reaction. The TaqMan™ Gene Expression Assay IDs (FAM) (Applied Biosystems, 4331182) are listed in *Table 5.2*. Taqman reactions were multiplexed with Human GAPD (GAPDH) Endogenous Control (VIC™/TAMRA™ probe, primer limited) (Thermo Fisher Scientific, 4326317E) to allow quantification of relative gene expression. The other type of qPCR setup was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, A25741) in accordance with the included product data sheet. 10 ng of cDNA was included within each reaction. 800 nM of forward and reverse primer was provided in each reaction. KiCqStart® SYBR® Green Primers (Sigma Aldrich, KSPQ12012) were used in all reactions and are listed in *Table 5.3*.

Table 5.2: Taqman Probes

Gene Name	Assay ID
ACTA2	Hs00426835_g1
COL1A1	Hs00164004_m1
GAPDH	Thermo Fisher Scientific, 4326317E

Table 5.3: SYBR Green PCR Primers

Gene Name	Sequence (5' – 3')	Tm (°C)
KCNA4	F – GGT GAA GTG CTT CTT CTG	55.7
	R – GGT AGA GAA GGT GCT AGA TG	55.5
KCNA5	F – ACT CTA AAT AGC CCA GAT CC	56.4
	R – ACT ACA GTA CAA AAA GCC AC	53.4
CACNA1C	F – GGA GAG TTT TCC AAA GAG AG	57.1
	R – TTT GAG ATC CTC TTC TAG CTG	57.9
TRPC4	F – AGG AAA TGA TCC GAC TAG ATG	59.6
	R – CTT GTC ATC ATT CCC TGT ATC	58.5
TRPM3	F – AGG TGT TAT TCG TCA TGT TG	57.5
	R – GCA ATA CCT ATG GTG CAT ATC	58.4
KCNJ2	F – GTC TTG GGA ATT CTG GTT TG	61.0
	R – GAA CAT GTC CTG TTG CTG	57.6
KCNMA1	F – CAT TTG GTG GAG AAT TCA GG	61.9
	R – GAT GAA GAA GAC CAT GAA GAG	57.9
PIEZO1	F – GAG GAT GTT CAA CCA GAA TG	59.5
	R – GGA AGA GGT TGA GAT GAT TG	58.7
PIEZO2	F – GGT TGT TTG GTA TCA AGT CAG	58.6
	R – AGA GTG TAG TAC ATC ACC AG	51.2

<i>TRPV4</i>	F – ACT TCA TCT ACT CTG TCC TG R – GAA GTA AAG GGC ATT CAT CC	53.1 60.0
<i>TRPM7</i>	F – CAA TAG AAG AAT GGT CTG TGG R – TAC TTA GCT CTG TAG GAA TGA G	58.7 54.6
<i>S100A4</i>	F – AAG TTC AAG CTC AAC AAG TC R – CAG CTT CAT CTG TCC TTT TC	56.1 59.0
<i>SCN5A</i>	F – GAA GAG TTA GAG GAG TCT CG R – TGA TGG TGA GGT CAG TAA AC	56.8 54.7
<i>SCN9A</i>	F – AAA GGG AAA ACA ATC TTC CG R – TGT ACT CGA CAT TTT TGG TC	61.8 57.6
<i>KCND2</i>	F – GCT ACA GTT ATG TTC TAC GC R – ATA CCC TAG TGT TGT CAT GG	54.2 56.3
<i>KCNB1</i>	F – AAG ATC CTT GCC ATA ATT TCC R – GAA CCT CAG CAG GTA CTC	60.7 54.5
<i>GAPDH</i>	F – GAT TCC ACC CAT GGC AAA TTC R – GTC ATG AGT CCT TCC ACG ATA C	54.6 54.8

5.2.2.4 qPCR Procedure and Analysis

All qPCRs were performed in either a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (Thermo Fisher Scientific, 4346906) or a MicroAmp™ Optical 384-Well Reaction Plate with Barcode (Thermo Fisher Scientific, 4309849). Plates were briefly spun down, prior to the initiation of the reaction. Reactions were performed in either an Applied Biosystems™ 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, 4351106) or QuantStudio™ 7 Flex Real-Time PCR System, 384-well, desktop (Thermo Fisher Scientific, 4485701). Melt Curves were performed at the end of reactions involving the use of PowerUp™ SYBR™ Green Master Mix to check the specificity of amplicon product. Analysis of qPCR data was performed using the Livak method outlined in Livak and Schmittgen, (2001). The housekeeping gene *GAPDH* was used to normalise gene expression in all qPCRs. Three technical replicates were performed for each sample.

5.2.3 RNA-Seq

5.2.3.1 RNA-Seq Experimental Conditions

The iPSC-CFs and ACFs were split into T25 flasks at a seeding density of 10,000 cells/cm² and cultured in Fibroblast Growth Medium 3 supplemented with 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 for 48 hours. At this point, the cells are approximately 50 % confluent. The medium was then changed with either Fibroblast Growth Medium 3 supplemented with 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) or 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media). After 12 hours of treatment, cells were dissociated and spun down. Dry pellets were frozen at – 80 °C. The experiment was performed using 4 biological replicates of iPSC-CFs and ACFs. A biological replicate was defined as cells separated from each other by at least one passage.

5.2.3.2 RNA Isolation and Quality

RNA was isolated from the frozen dry pellets as described in 5.2.2.1. Purity of RNA was assessed using a NanoDrop 2000. Thresholds of 1.8- 2.0 for the 260/280 ratio and 2.0-2.2 for the 260/230 were imposed on samples. RNA was run on an Agilent 2200 TapeStation for characterisation and quality assessment. All samples used for RNA-seq had a measured RNA integrity number (RIN) of greater than 9.5.

5.2.3.3 Library Preparation, Sequencing and Analysis

RNA samples were sent to EuroFins for INVIEWS Transcriptome Discover library preparation, sequencing, and analysis. Library preparation consisted of purification of poly-A containing mRNA molecules, mRNA fragmentation, random primed cDNA synthesis (strand-specific), adapter ligation and adapter specific PCR amplification. Illumina paired-

end read sequencing (2 x 150 bp) was performed with a guaranteed depth of 30 M read pairs (+/- 3%). RNA sequencing analysis was performed by EuroFins and consisted of mapping against one reference genome and identification and quantification of transcripts. Gene counts were provided by EuroFins. All further analysis was performed using iDEP (Ge et al., 2018). Further analysis consisted of removing genes with minimal counts per million of less than 30 in at least 1 sample library. Counts data was prepared for clustering, K-means analysis and PCA by applying a regularized log scale transformation. A strict threshold for differentially expressed genes was applied, consisting of a false discovery rate (FDR) < 0.01 and a minimum fold change of 2. Pathway analysis was performed using the GO Biological Process function.

5.2.4 Quantitative Proteomics

5.2.4.1 *Quantitative Proteomics Experimental Conditions*

The iPSC-CFs and ACFs were split into T25 flasks at a seeding density of 10,000 cells/cm² and cultured in Fibroblast Growth Medium 3 (FGM3) supplemented with 10 µM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 Media for 48 hours. At this point, the cells were approximately 50 % confluent. The media was then changed with FGM3 with either 10 µM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) or 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (Activation Media). After 48 hours of treatment, cells were dissociated and spun down. Dry pellets were frozen at – 80 °C. The experiment was performed using 5 biological replicates of iPSC-CFs and ACFs. A biological replicate was defined as cells separated from each other by at least one passage.

5.2.4.2 Quantitative Proteomics Sample Preparation (Lysis, Digestion and Labelling)

The lysis and digestion of protein samples was carried out using an EasyPep™ MS Sample Prep Kit (Thermo Fisher Scientific, A40006). Lysis and digestion were carried out according to the manufacturer's protocol apart from when stipulated below. Quantification and normalisation of protein samples was carried out prior to digestion into peptide.

Quantification of protein samples was carried out using Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific, A53225), Pierce™ Bovine Serum Albumin Standard Ampules, 2 mg/mL (Thermo Fisher Scientific, 23209) and a SpectraMax ABS Microplate Reader (VWR, 736-0916). Quantification was performed according to the manufacturer's protocol. Following quantification, 40 µg of each protein sample was used for digestion and labelling. Digestion was performed contrary to the protocol for 16 hours at 37 °C (150 rpm). Labelling of samples was performed using a TMT10plex™ Isobaric Label Reagents and Kits (Thermo Fisher Scientific, 90110) according to the protocol outlined in the EasyPep™ MS Sample Prep Kit. Peptide clean-up was carried out following labelling using the peptide clean-up columns included in the kit. A member of staff from the Advanced Mass Spectrometry Facility Services (University of Birmingham) combined the samples and performed a final ZipTip clean-up (Merck Millipore, ZTC18S096).

5.2.4.3 Mass Spectrometry

Samples were run on a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) coupled with an UltiMate® 3000 HPLC series (Dionex). Samples were trapped on a Precolumn Cartridge, Acclaim PepMap 100 C18, 5 µm 100 Å, 300 µm i.d. x 5 mm (Dionex) and separated in a Nano Series™ Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex). The gradient used was from 3.2 % to 24 % solvent B (0.1 % formic acid in acetonitrile) for 30 mins followed by an increase to 60 %

for 15 min. The column was washed with 90 % solvent B solution for 15 mins and equilibrated with 3.2 % of solvent B for a further 15 mins. The total run time was 75 mins. Peptides were eluted directly ($\sim 350 \text{ nL min}^{-1}$) via a Triversa Nanomate nanospray source (Advion Biosciences) into a QEHF mass spectrometer. The data-dependent scanning acquisition was controlled by Xcalibur 4.0 software. The mass spectrometer alternated between a full FT-MS scan (m/z 375 – 1600) and high energy collision dissociation (HCD) MS/MS scans of the 15 most abundant ions. Survey scans are acquired in the QEHF with a resolution of 120,000 at m/z 200 and automatic gain control (AGC) 3×10^6 . Precursor ions were fragmented in HCD MS/MS with a resolution set up at 60,000 and a normalized collision energy of 32. The AGC target for HCD MS/MS was 1×10^5 . The width of the precursor isolation window was 1.2 m/z and only multiply charged (2-6) precursor ions were selected for MS/MS. Spectra were acquired for 60 mins with a dynamic exclusion time of 20s. The MS and MS/MS scans are searched against Uniprot database using Proteome Discoverer 2.2 (Thermo Fisher Scientific) with a 1% false discovery rate (FDR). Oxidation (M) and N terminal acetylation are set as variable modifications. Cysteine carbamidomethylation and TMT 6-plex on lysine and N-terminals of the peptide as fixed modifications. The precursor mass tolerance was set at 10 ppm and a MS/MS mass tolerance of 0.02 Da for reporter ions quantification.

5.2.5 Statistical Analysis

All statistics, apart from RNA-Seq and quantitative proteomics, was performed using GraphPad Prism Version 10.0.03. Gaussian distribution was tested using a Shapiro-Wilk normality test. Either an unpaired t-test with a Welch's correction or a one-way ANOVA with Tukey post-hoc analysis was used to test significance and taken as $p < 0.05$.

5.3 Results

5.3.1 Generation of iPSC-CFs

Cardiac fibroblasts were differentiated from iPSCs using a modified protocol from Zhang et al., (2019). The cells derived demonstrated morphological features similar to primary cardiac fibroblasts, namely a flat and spindle like shape. Bright field images of the cells derived are shown in *Figure 5.2*. The cells derived were split into geltrex coated 24 well plates and fixed for immunofluorescence. The cells stained positive for the cardiac fibroblast marker vimentin as shown in *Figure 5.3*.

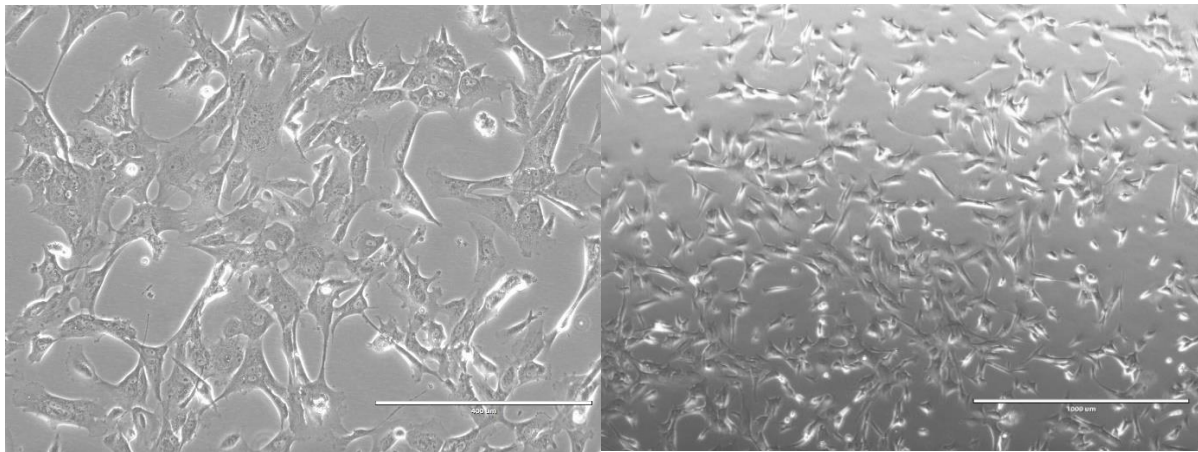


Figure 5.2: Bright field images of iPSC-CFs

Images of iPSC-CFs were taken using a bright field microscope at x 20 (left) and x 10 (right) magnification. The cells demonstrate morphological features of primary cardiac fibroblasts. Left Scale bar = 400 μm. Right Scale bar = 1000 μm.

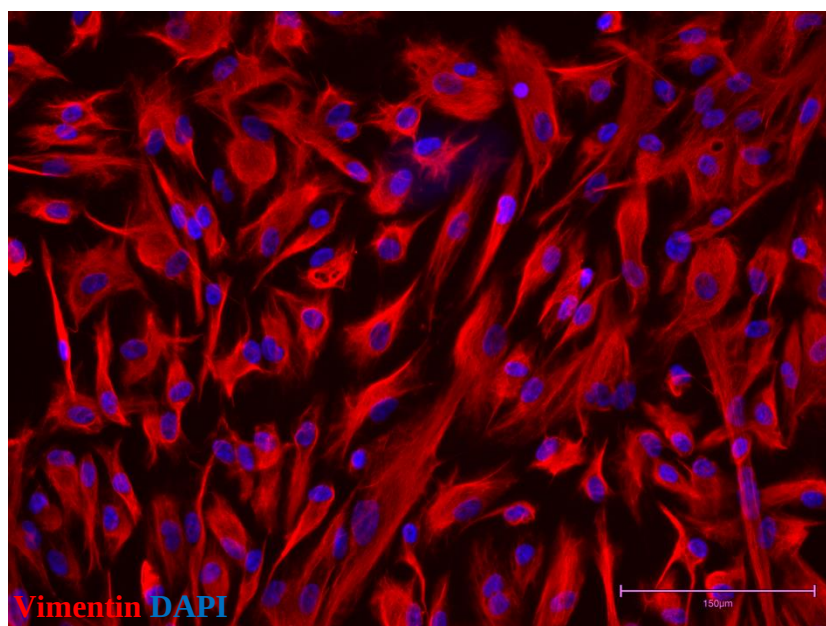


Figure 5.3: Immunofluorescence of iPSC-CFs

iPSC-CFs were fixed and stained for vimentin (red) and DAPI (blue). Scale bar = 150 μ m.

5.3.2 Activation of iPSC-CFs, VCFs and ACFs

5.3.2.1 Immunofluorescence of Activation Markers in iPSC-CFs, VCFs and ACFs

Experiments were performed to assess the plasticity of iPSC-CFs, VCFs and ACFs for myofibroblast activation. The cells were passaged into geltrex coated wells of a 24 well plate and cultured for 72 hours in fibroblast growth media 3 supplemented with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media). The cells were fixed and stained for vimentin, α SMA, collagen 1 and DAPI (nuclei). α SMA is a marker expressed by activated cardiac fibroblasts or myofibroblasts, whilst vimentin and collagen 1 are markers expressed by both cardiac fibroblasts and myofibroblasts. Expression of collagen 1 should increase with cardiac fibroblast activation. The images of the iPSC-CFs, VCFs and ACFs are respectively shown in *Figures 5.4, 5.5 and 5.6*. The iPSC-CFs demonstrated a dramatic increase in expression of α SMA and collagen following treatment with 10 ng/mL TGF- β 1. The VCFs showed

expression of α SMA when cultured in media containing 10 μ M of the TGF- β 1 inhibitor SB431542. Significantly greater expression of α SMA and collagen 1 was observed in the VCFs following treatment with 10 ng/mL TGF- β 1. The ACFs demonstrated minimal increases in collagen 1 and α SMA expression following TGF- β 1 treatment. Overall, the iPSC-CFs and VCFs showed the greatest activation following TGF- β 1 treatment. The ACFs showed nearly no expression of α SMA following treatment with TGF- β 1.

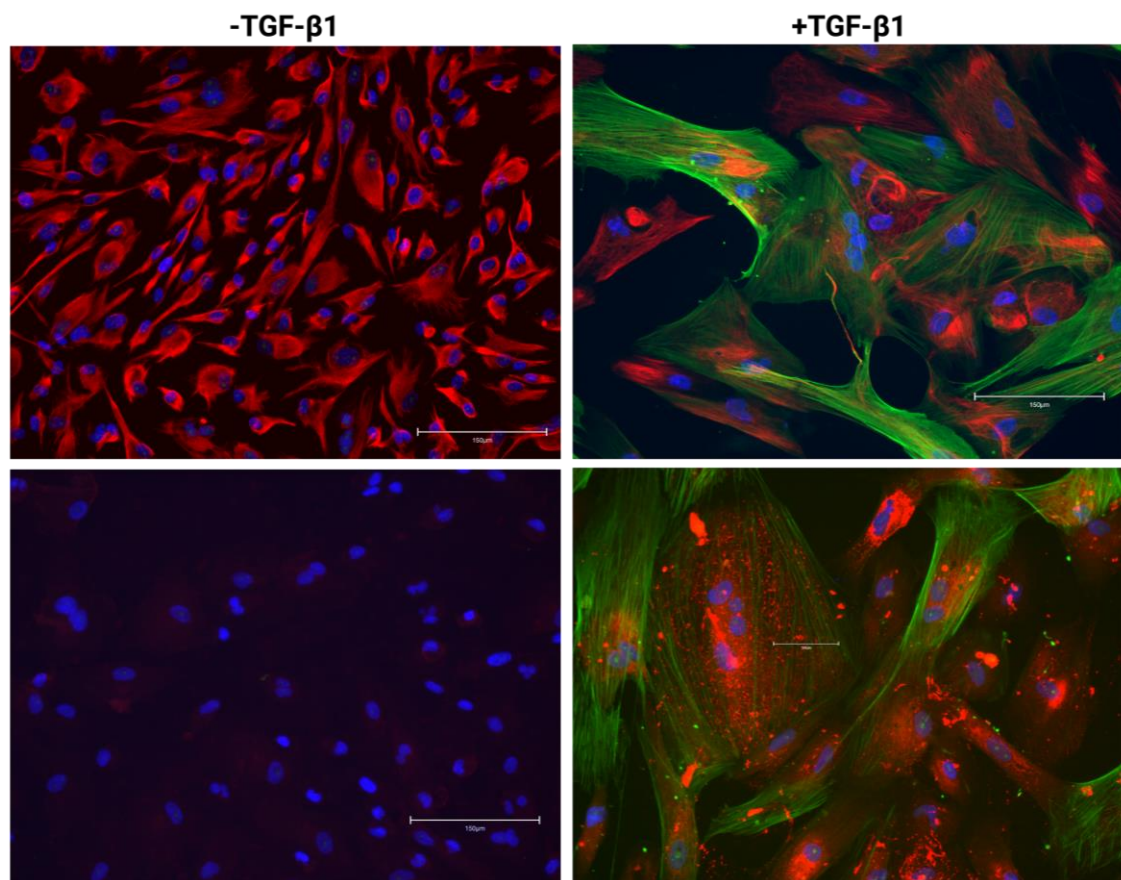


Figure 5.4: Activation of iPSC-CFs

iPSC-CFs were treated for 72 hours in fibroblast growth media 3 supplemented with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (+ **TGF- β 1**) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (- **TGF- β 1**). The cells were fixed and stained for vimentin (red), α SMA (green) and DAPI (blue) (**top**) and collagen 1 (red), α SMA (green) and DAPI (blue) (**bottom**). Scale bar = 150 μ m.

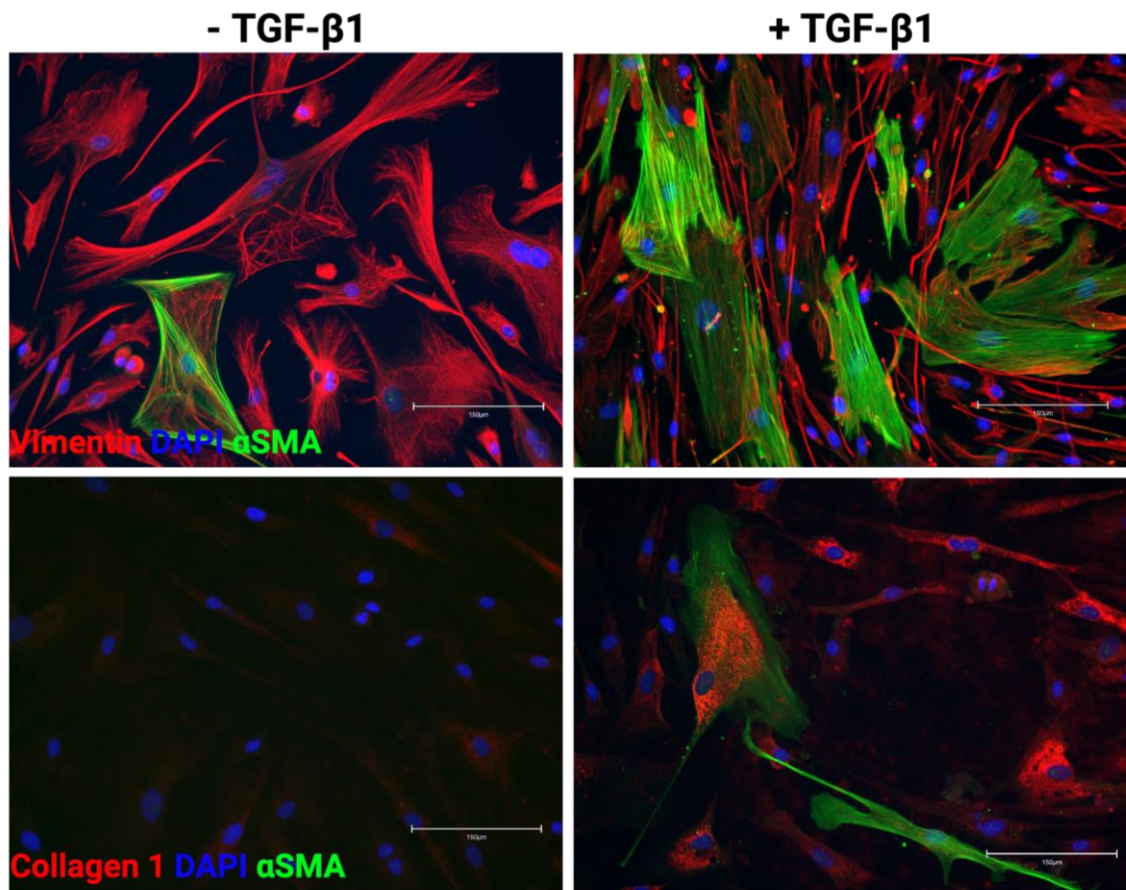


Figure 5.5: Activation of VCFs

VCFs were treated for 72 hours in fibroblast growth media 3 supplemented with either 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (+ **TGF-β1**) or 10 μM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (- **TGF-β1**). The cells were fixed and stained for vimentin (red), αSMA (green) and DAPI (blue) (**top**) and collagen 1 (red), αSMA (green) and DAPI (blue) (**bottom**). Scale bar = 150 μm.

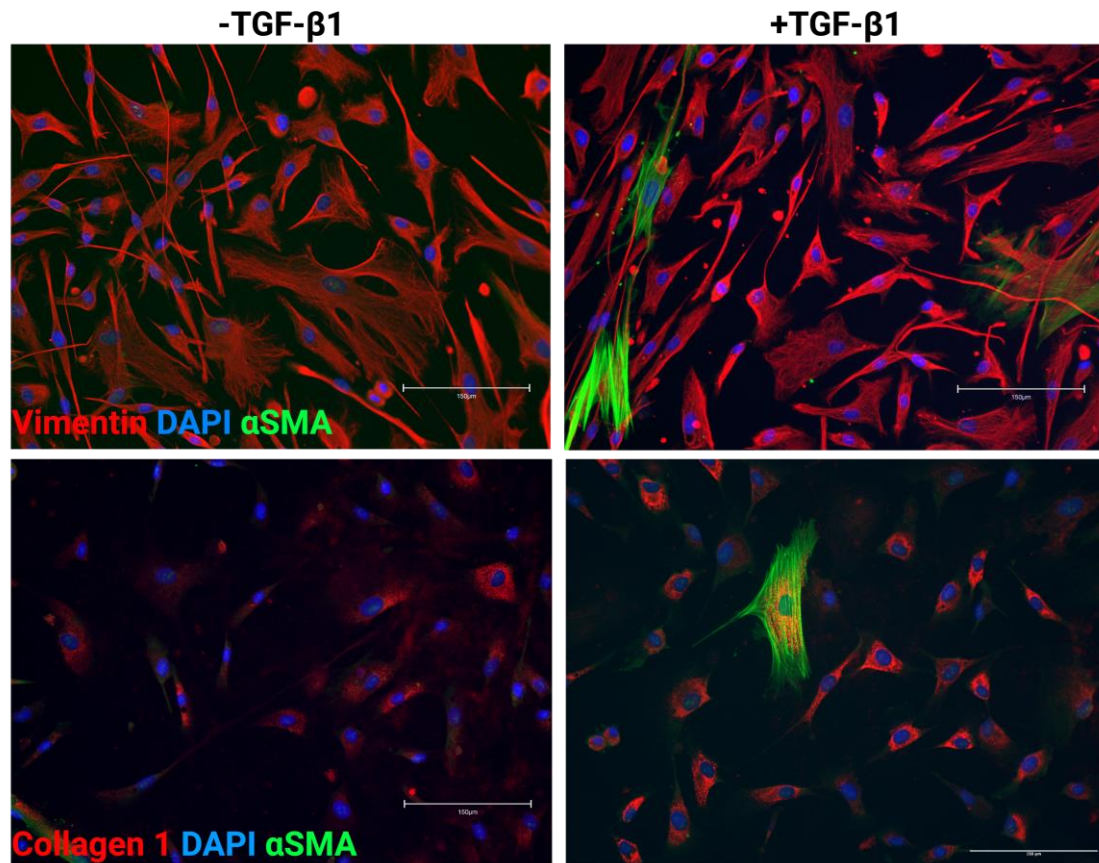


Figure 5.6: Activation of ACFs

ACFs were treated for 72 hours in fibroblast growth media 3 supplemented with either 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (+ **TGF-β1**) or 10 μM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (- **TGF-β1**). The cells were fixed and stained for vimentin (red), αSMA (green) and DAPI (blue) (**top**) and collagen 1 (red), αSMA (green) and DAPI (blue) (**bottom**). Scale bar = 150 μm.

5.3.2.2 Gene Expression

To assess whether the cells could be activated, the iPSC-CFs, VCFs and ACFs were passaged into geltrex coated T25 flasks at 10,000 cells/cm² in either media containing 10 μM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) or 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (Activation Media). The media on the cells was changed 24 hours after passaging and the cells were cultured for a further 48 hours (72 hours in total). This was performed on 3 biological replicates for the iPSC-CFs and ACFs and 2 biological replicates for the VCFs. A biological replicate was considered as cells separated by at least one passage. qPCRs were performed for activation markers *COL1A1* (Collagen 1) and *ACTA2* (αSMA)

and are shown in *Figure 5.7*. All three cell lines showed a significant upregulation in *COL1A1* and *ACTA2* expression following TGF- β 1 treatment. The iPSC-CFs demonstrated the lowest expression of *COL1A1* when cultured in the quiescent media. Expression of *COL1A1* in iPSC-CFs increased approximately 3-fold following TGF- β 1 treatment. The expression of *COL1A1* in VCFs increased approximately 10-fold following TGF- β 1 treatment. ACFs demonstrated the greatest expression of *COL1A1* when cultured in quiescent media. Expression increased approximately 2-fold following TGF- β 1 treatment. Expression of *ACTA2* expression increased the most following TGF- β 1 treatment in the iPSC-CFs with a 5-fold increase. The VCFs and ACFs demonstrated a 3-fold and 1.5-fold increase in *ACTA2* expression following TGF- β 1 treatment.

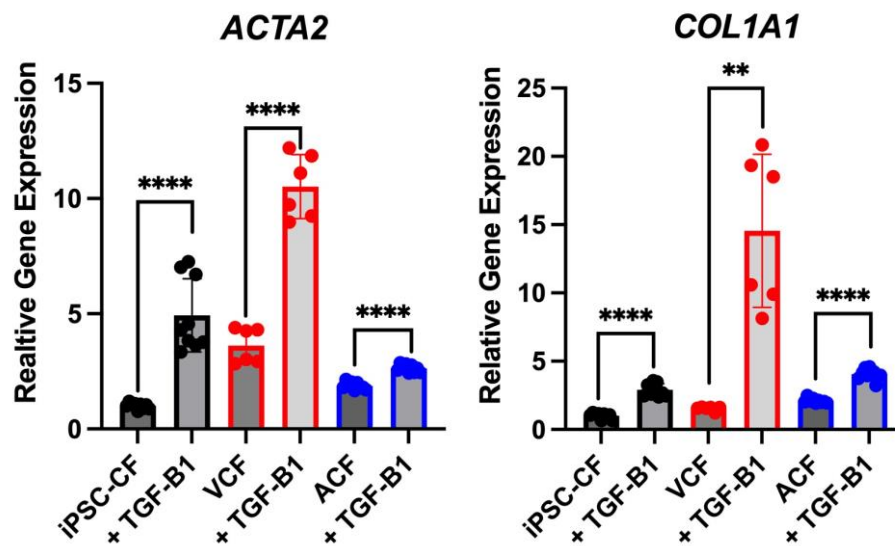


Figure 5.7: Activation of iPSC-CFs, VCFs and ACFs

iPSC-CFs, VCFs and ACFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) (+ **TGF- β 1**) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for activation markers *COL1A1* (Collagen 1) and *ACTA2* (α SMA). iPSC-CFs (N = 3, n = 9), VCFs (N = 2, n = 6), ACFs (N = 3, n = 9). Gene expression was normalised to *GAPDH*. A Welch's t test was used to ascertain significance (* denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001, **** denotes P < 0.0001).

5.3.3 Electrophysiological Gene Changes in iPSC-CFs and VCFs

Having demonstrated varying degrees of plasticity in all three cardiac fibroblast cell lines, experiments were undertaken to determine the effect TGF- β 1 had on ion channel subunit gene expression in iPSC-CFs. Ion flux is pivotal in the initial activation of cardiac fibroblasts to myofibroblasts and in the subsequent pathophysiological depolarisation of the membrane potential following activation. As the VCFs showed greater plasticity than the ACFs, they were determined to be a better model for comparing gene expression during cardiac fibroblast activation.

The iPSC-CFs and VCFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for genes predicted to be important in the electrophysiological changes that occur as part of and because of cardiac fibroblast activation. The genes assessed include those that encode transient receptor proteins (TRPs): *TRPM3*, *TRPM7*, *TRPV4*, *TRPC4*. Voltage gated ion channel subunit genes: *KCNJ2*, *KCNA5*, *KCNMA1*, *CACNA1C*, *SCN5A*, *SCN9A*, *KCNB1*, *KCND2*, *KCNA4*. Genes encoding piezo channels and fibroblast specific protein: *PIEZO1*, *PIEZO2*, *S1000A4*.

5.3.3.1 Transient Receptor Protein Channel Expression

The gene expression of *TRPM3*, *TRPM7*, *TRPV4* and *TRPC4* in iPSC-CFs and VCFs is shown in *Figure 5.8*. The iPSC-CFs demonstrated no significant difference in *TRPM3*, *TRPV4* and *TRPC4* expression following treatment with 10 ng/mL TGF- β 1. The iPSC-CFs did however show a significant decrease in *TRPM7* expression. Expression of all the TRP genes assayed was consistently greater in the VCFs, with *TRPM3* being expressed

approximately 200-fold more in the primary cells. The VCFs showed a significant decrease in *TRPM3* and *TRPV4* expression following treatment with 10 ng/mL TGF- β 1.

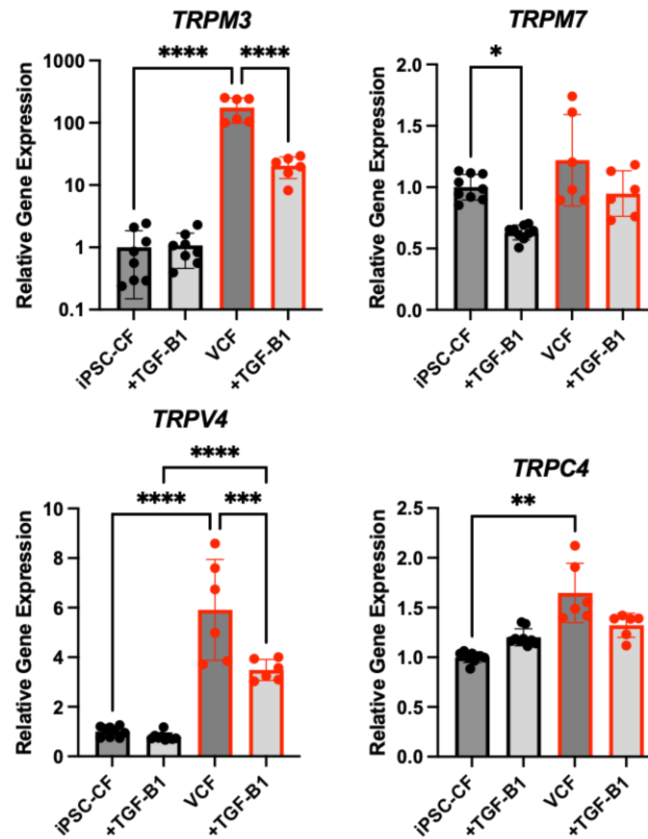


Figure 5.8: TRP Channel Gene Expression in iPSC-CFs and VCFs

iPSC-CFs and VCFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) (+ **TGF- β 1**) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for *TRPM3*, *TRPM7*, *TRPV4* and *TRPC4*. iPSC-CFs (N = 3, n = 9), VCFs (N = 2, n = 6). Gene expression was normalised to *GAPDH*. A nested Tukey two-way ANOVA was used to ascertain significance (* denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001, **** denotes P < 0.0001).

5.3.3.2 Voltage Gated Ion channel Expression

The gene expression of the voltage gated ion channel subunits *SCN5A*, *SCN9A*, *CACNA1C*, *KCND2*, *KCNB1*, *KCNJ2* and *KCNMA1* was measured in iPSC-CFs and VCFs and is shown in Figure 5.9. The expression levels of *KCNA5* and *KCNA4* were low enough to indicate physiological irrelevance (Ct values > 32). Expression of the inward sodium current channel subunit genes *SCN5A* and *SCN9A* was significantly greater in the iPSC-CFs than in the

VCFs. The expression of *SCN5A* significantly increased in the iPSC-CFs following TGF- β 1 treatment. Conversely, the VCFs demonstrated a decrease in expression of *SCN5A* following TGF- β 1 treatment. Both the iPSC-CFs and VCFs demonstrated robust expression of the L-type calcium channel ion channel subunit gene *CACNA1C*. Expression was increased in both cell types following treatment with TGF- β 1; however the increase was not statistically significant in the iPSC-CFs.

Similar levels of expression of the transient outward potassium current gene *KCND2* were detected in the iPSC-CFs and VCFs. Both cardiac fibroblast cell lines demonstrated a significant decrease in expression following TGF- β 1 treatment. *KCNB1* encodes the K_v2.1 potassium channel, which has a variety of roles including regulating Ca²⁺ channel function and membrane potential. Both the iPSC-CFs and VCFs demonstrated a significant decrease in expression of *KCNB1* following TGF- β 1 treatment. The inwardly rectifying potassium channel K_{ir}2.1 is encoded by *KCNJ2* and plays an important role in the depolarisation of cardiac fibroblasts during cardiac fibroblast activation. Increased expression of *KCNJ2* was observed in both the iPSC-CFs and VCFs, however it was only statistically significant in the VCFs. *KCNAM1* encodes a subunit of the large conductance potassium channel (BK_{Ca}), which can be activated by changes in intracellular Ca²⁺ or membrane potential and can promote cardiac myofibroblast differentiation. Expression of *KCNMA1* increased in both cardiac fibroblast cell lines following TGF- β 1 treatment but was not statistically significant.

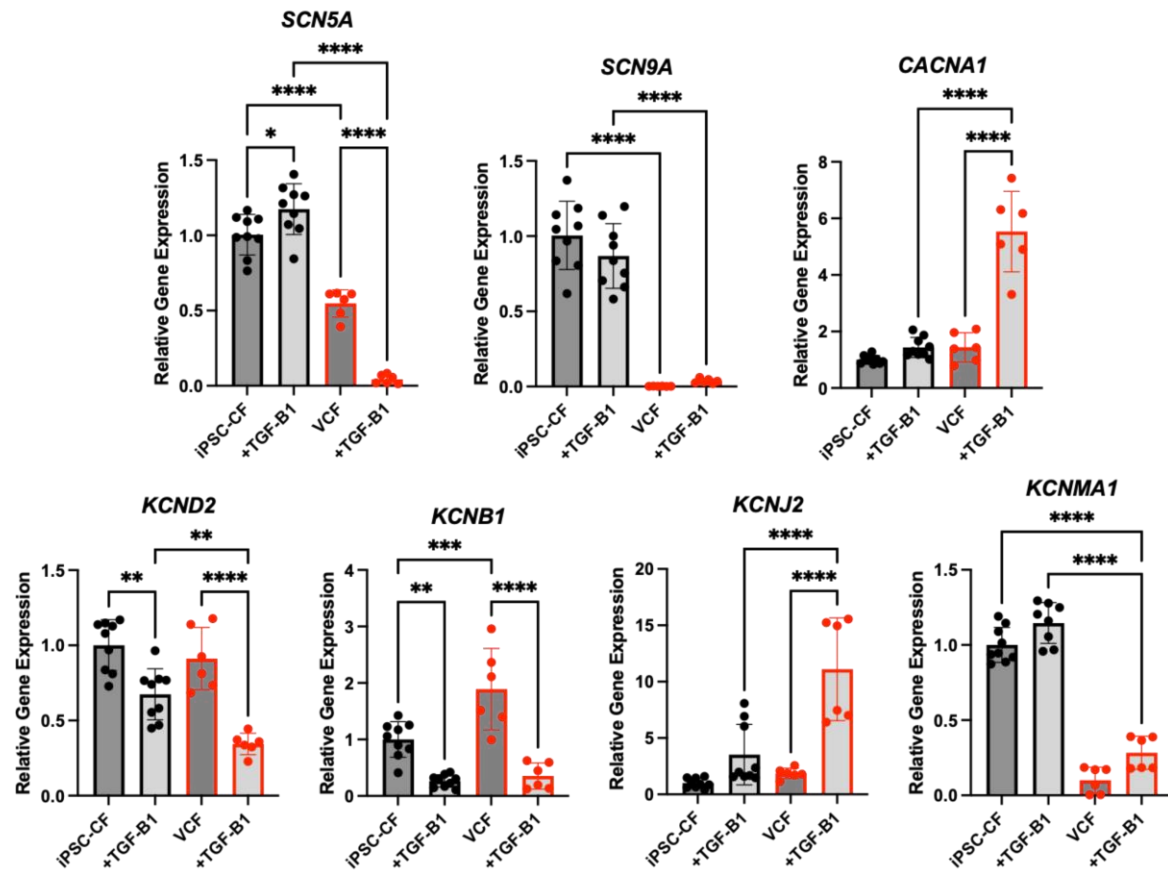


Figure 5.9: Voltage-Activated Ion Channel Subunit Gene Expression in iPSC-CFs and VCFs

iPSC-CFs and VCFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) (+ TGF- β 1) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for *SCN5A*, *SCN9A*, *CACNA1C*, *KCND2*, *KCNB1*, *KCNJ2* and *KCNMA1*. iPSC-CFs (N = 3, n = 9), VCFs (N = 2, n = 6). Gene expression was normalised to *GAPDH*. A nested Tukey two-way ANOVA was used to ascertain significance (* denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$, **** denotes $P < 0.0001$).

5.3.3.3 Piezo Channels and *S100A4* Expression

The piezo channels, whose name comes from the Greek word to squeeze or press, are a group of mechano-sensitive ion channels expressed in cardiac fibroblasts. Overexpression of piezo channels is broadly associated with myocardial remodelling and stretch-mediated activation of cardiac fibroblasts. *S100A4*, also known as fibroblast specific protein 1 (FSP1), encodes a calcium binding protein broadly associated with cardiac remodelling and fibrosis. Expression of *PIEZO1*, *PIEZO2* and *S100A4* (FSP1) in iPSC-CFs and VCFs is shown in *Figure 5.10*.

Expression of *S100A4* was approximately 17-fold greater in the VCFs than the iPSC-CFs. No

significant differences in expression were observed in the iPSC-CFs following TGF- β 1 treatment. The VCFs demonstrated a significant decrease in *S100A4* gene expression. No significant differences in expression were seen in *PIEZO1* in and between the iPSC-CFs and VCFs. Expression of *PIEZO2* showed a significant increase in the iPSC-CFs following TGF- β 1 treatment. Conversely expression decreased in the VCFs.

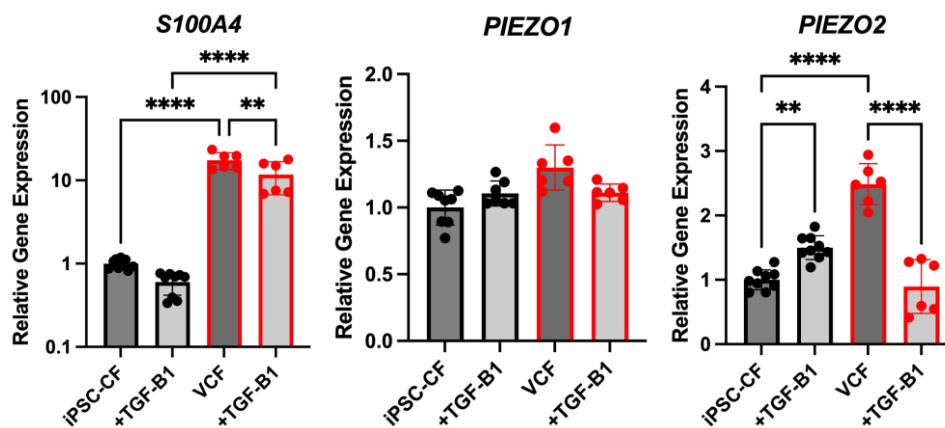


Figure 5.10: *S100A4*, *PIEZO1* and *PIEZO2* Gene Expression in iPSC-CFs and VCFs

iPSC-CFs and VCFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) (+ **TGF- β 1**) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for *S100A4*, *PIEZO1* and *PIEZO2*. iPSC-CFs (N = 3, n = 9), VCFs (N = 2, n = 6). Gene expression was normalised to *GAPDH*. A nested Tukey two-way ANOVA was used to ascertain significance (* denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001, **** denotes P < 0.0001).

5.3.4 Electrophysiological Gene Changes in iPSC-CFs, VCFs and ACFs

The iPSC-CFs and VCFs demonstrated a broadly similar response to TGF- β 1 treatment in the expression of the following genes: *TRPM7*, *TRPV4*, *CACNA1C*, *KCND2*, *KCNB1*, *KCNJ2*, *KCNMA1* and *S100A4* as seen in Table 5.4. Despite this, the gene expression of several genes thought to be integral to cardiac fibroblast activation differed following TGF- β 1 treatment (*PIEZO2*, *TRPC4*, *TRPM3*). Furthermore, some genes that were expected to be upregulated following TGF- β 1 treatment demonstrated either no change in gene expression or downregulation in the iPSC-CFs and VCFs (*S100A4*, *PIEZO1*, *TRPM7*, *TRPV4*, *TRPC4*).

To resolve this discrepancy, the ACFs were also investigated. This was performed to understand whether the previous results were consistent in another cardiac fibroblast cell line. ACFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for *KCNMA1*, *CACNA1C*, *S100A4*, *TRPM7*, *TRPC4*, *PIEZO1* and *TRPV4*. The results are shown in *Figure 5.11*. The ACFs demonstrated no significant change in expression of *CACNA1C*, *KCNMA1* and *S100A4* following TGF- β 1 treatment. Expression of *PIEZO1* and *TRPV4* significantly increased in the ACFs following TGF- β 1 treatment. The cells however showed no significant difference in *TRPC4* and *TRPM7* expression following TGF- β 1 treatment. Following completion of these experiments, it was ascertained that the VCFs were derived from an explanted heart, making them a potentially unphysiological primary control. The ACFs were used for future experiments as a primary control for the iPSC-CFs.

Table 5.4: Comparison of Gene Expression in iPSC-CFs and VCFs

Gene	Fold Change in Expression Following TGF- β 1 Treatment (P Value)	
	iPSC-CFs	VCFs
<i>TRPM3</i>	1.08 (0.99)	0.13 (0.0001)
<i>TRPM7</i>	0.63 (0.57)	0.78 (0.92)
<i>TRPV4</i>	0.79 (0.99)	0.58 (0.0015)
<i>TRPC4</i>	1.20 (0.99)	0.80 (0.99)
<i>SCN5A</i>	1.17 (0.0405)	0.078 (0.0001)
<i>SCN9A</i>	0.87 (0.36)	8.75 (0.99)
<i>CACNA1C</i>	1.44 (0.707)	3.84 (0.0001)
<i>KCND2</i>	0.67 (0.0014)	0.37 (0.0001)

<i>KCNB1</i>	0.26 (0.0019)	0.19 (0.0001)
<i>KCNJ2</i>	3.52 (0.17)	5.97 (0.0001)
<i>KCNMA1</i>	1.15 (0.0837)	2.8 (0.0638)
<i>S100A4</i>	0.60 (0.77)	0.68 (0.0016)
<i>PIEZO1</i>	1.11 (0.99)	0.85 (0.99)
<i>PIEZO2</i>	1.50 (0.0027)	0.36 (0.0001)

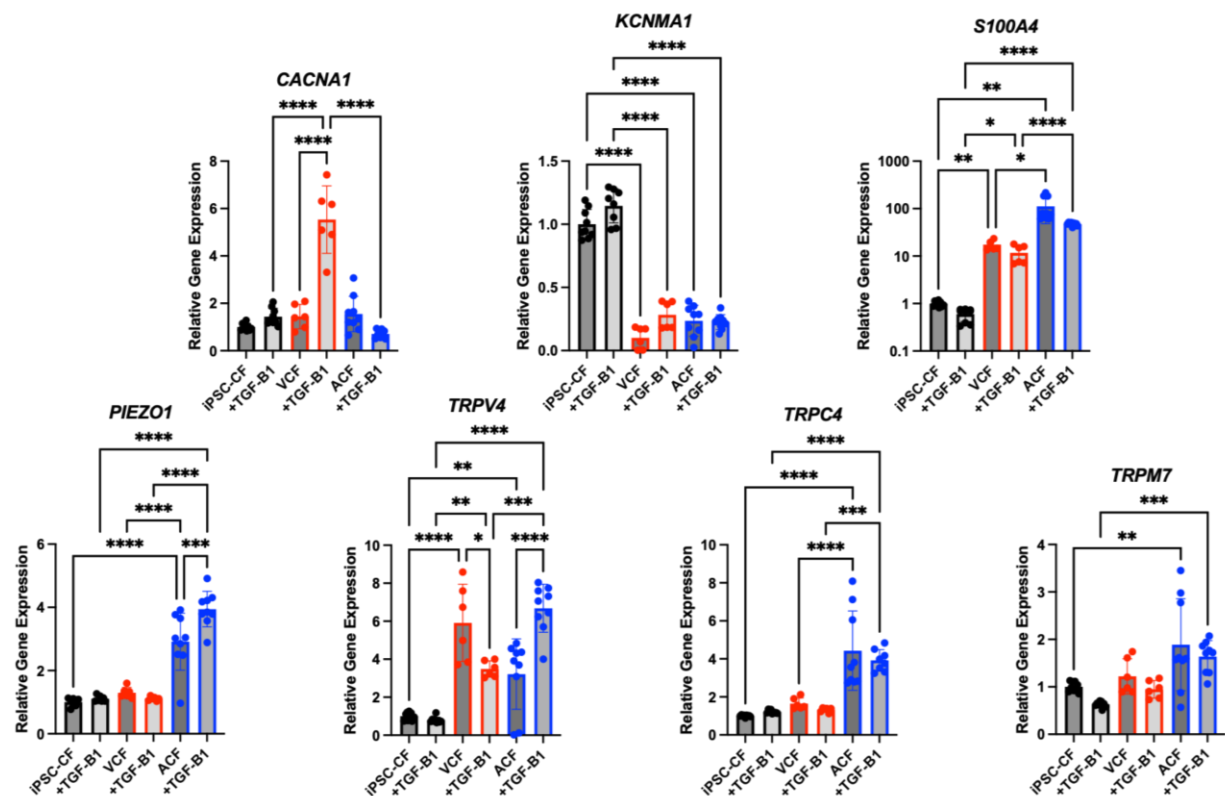


Figure 5.11: Cardiac Activation Gene Expression in iPSC-CFs, VCFs and ACFs

iPSC-CFs, VCFs and ACFs were treated with either 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (Activation Media) (+ TGF-β1) or 10 μM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 72 hours. The cells were spun down and pellets were harvested. RNA was extracted and converted into cDNA. qPCRs were performed for *S100A4*, *PIEZO1* and *PIEZO2*. iPSC-CF (N = 3, n = 9), VCFs (N = 2, n = 6), ACFs (N = 3, n = 9). Gene expression was normalised to *GAPDH*. A nested Tukey two-way ANOVA was used to ascertain significance (* denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$, **** denotes $P < 0.0001$).

5.3.5 RNA-Sequencing Analysis of iPSC-CFs and ACFs

5.3.5.1 *Experimental Design*

It became abundantly clear that the number of genes involved in cardiac fibroblast activation was too great to adequately assess using a low throughput approach like qPCR. Furthermore, a non-biased approach would better enable the accurate identification of ion channel subunit genes integral to myofibroblast differentiation and electrophysiology. Bulk RNA-sequencing (RNA-seq) was performed on iPSC-CFs and ACFs with and without TGF- β 1 treatment. In this way, the transcriptomic profiles of iPSC-CFs and ACFs could be compared to investigate changes in gene expression that occur during cardiac fibroblast activation.

The iPSC-CFs and ACFs were treated with either 10 ng/mL TGF- β 1 and 10 ng/mL FGF2 (Activation Media) (+ TGF- β 1) or 10 μ M TGF- β 1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) (- TGF- β 1) for 12 hours. 4 biological replicates, differing by at least 1 passage, were used for each condition (Cell type, +/- TGF- β 1). The cells were spun down and pellets were harvested. RNA was extracted. A 12-hour time point was chosen to ensure that changes in gene expression integral to the activation of cardiac fibroblast were detected. RNA was sent to EuroFins for library preparation, which consisted of purification of poly-A containing mRNA molecules, mRNA fragmentation, random primed cDNA synthesis (strand-specific), adapter ligation and adapter specific PCR amplification. Following this, EuroFins carried out Illumina paired-end read sequencing (2 x 150 bp, Guaranteed 30 M read pairs, +/- 3%).

5.3.5.2 *iPSC-CFs and ACFs demonstrate differential Gene Expression*

A principal component analysis (PCA) performed on the 4 samples (iPSC-CFs, iPSC-CFs-TGF, ACFs, ACFs-TGF) demonstrated demarcated expression profiles, as shown in *Figure 5.12*. The greatest differences in gene expression between the samples was due to cardiac

fibroblast cell type (iPSC-CFs vs ACFs). The RNA-seq analysis revealed 1524 differentially expressing genes (DEGs) between the iPSC-CFs and ACFs (780 upregulated, 744 downregulated). A heatmap, as well as some of the pathways that were up and downregulated in iPSC-CFs is shown in *Figure 5.13*. The iPSC-CFs were downregulated for the following gene pathways: blood cell development, cell adhesion, cell population proliferation, biological adhesion and extracellular matrix organisation. The iPSC-CFs were upregulated for the following gene pathways: cell to cell adhesion, anatomical structure morphogenesis, tissue development, nervous system development and cell differentiation. The top 50 differentially expressed genes are shown in *Figure 5.14*. Although expression of collagen encoding genes was broadly upregulated in the ACFs, the cells demonstrated differential expression of certain isoforms as shown in *Figure 5.15*. Substantial expression of *COL4A6* and *COL11A1* was observed in the iPSC-CFs, with nearly no expression in ACFs. The ACFs showed significantly greater expression of *COL1A2* and *COL6A3*.

A K-Means cluster analysis aims to partition groups of upregulated and downregulated genes into clusters based on gene function. It was performed on the top 100 most variable genes between the iPSC-CFs and ACFs and identified an upregulation of cell adhesion genes in iPSC-CFs and extracellular matrix organisation genes in ACFs. There was only two differentially expressed ion channel subunit genes in the top 1000 most variable genes (*KCNMA1* and *TRPV2*). Consistent with the qPCR data in *Figure 5.11*, *KCNMA1* showed significantly greater expression in the iPSC-CFs. *TRPV2* showed greater expression in the ACFs.

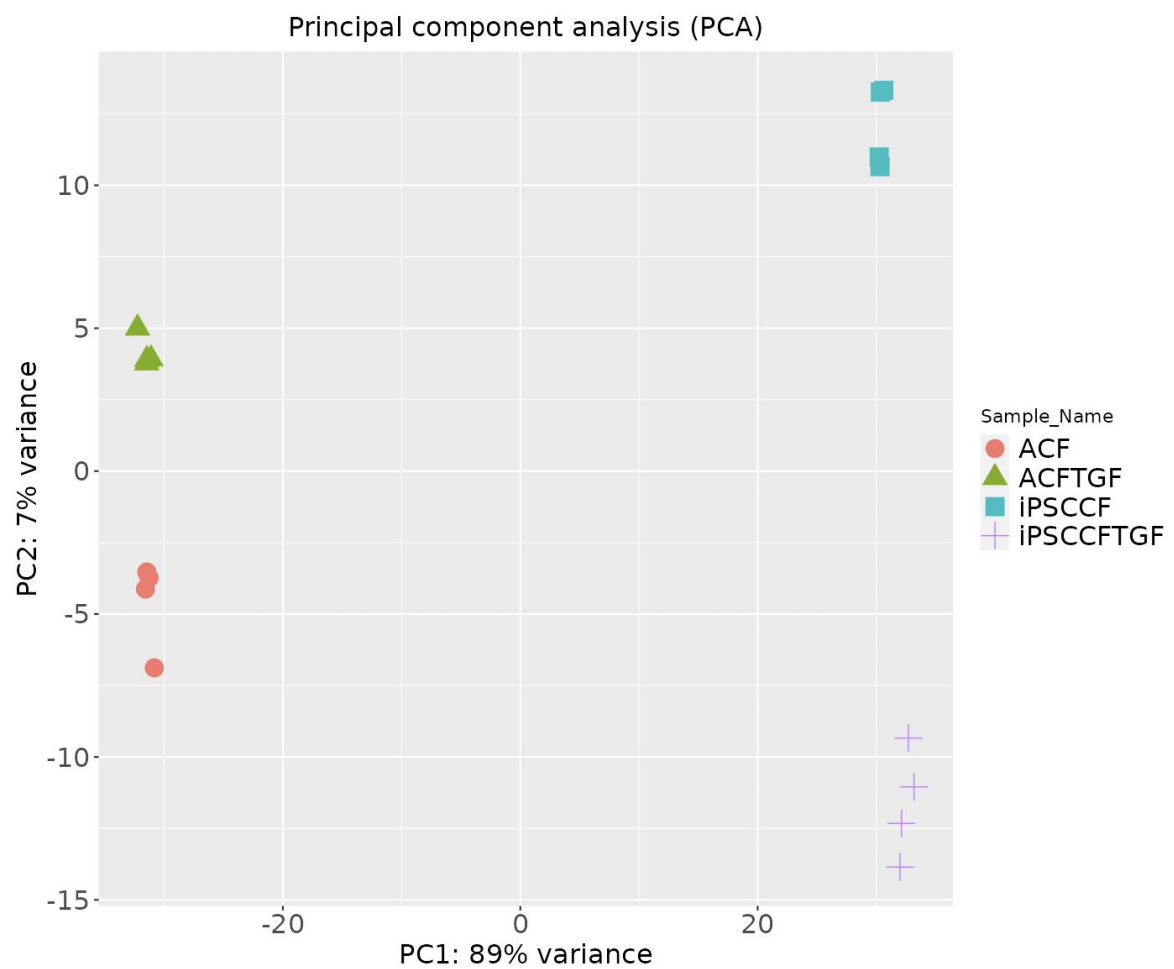


Figure 5.12: PCA Plot of iPSC-CFs and ACFs with and without TGF- β 1 Treatment

A PCA plot showing the first and second principal components. There is little variation between the biological replicates of the samples. There is large variation in gene expression between iPSC-CFs and ACFs. Treatment with 10 ng/mL TGF- β 1 for 12 hours caused a greater change in gene expression in the iPSC-CFs than in the ACFs.

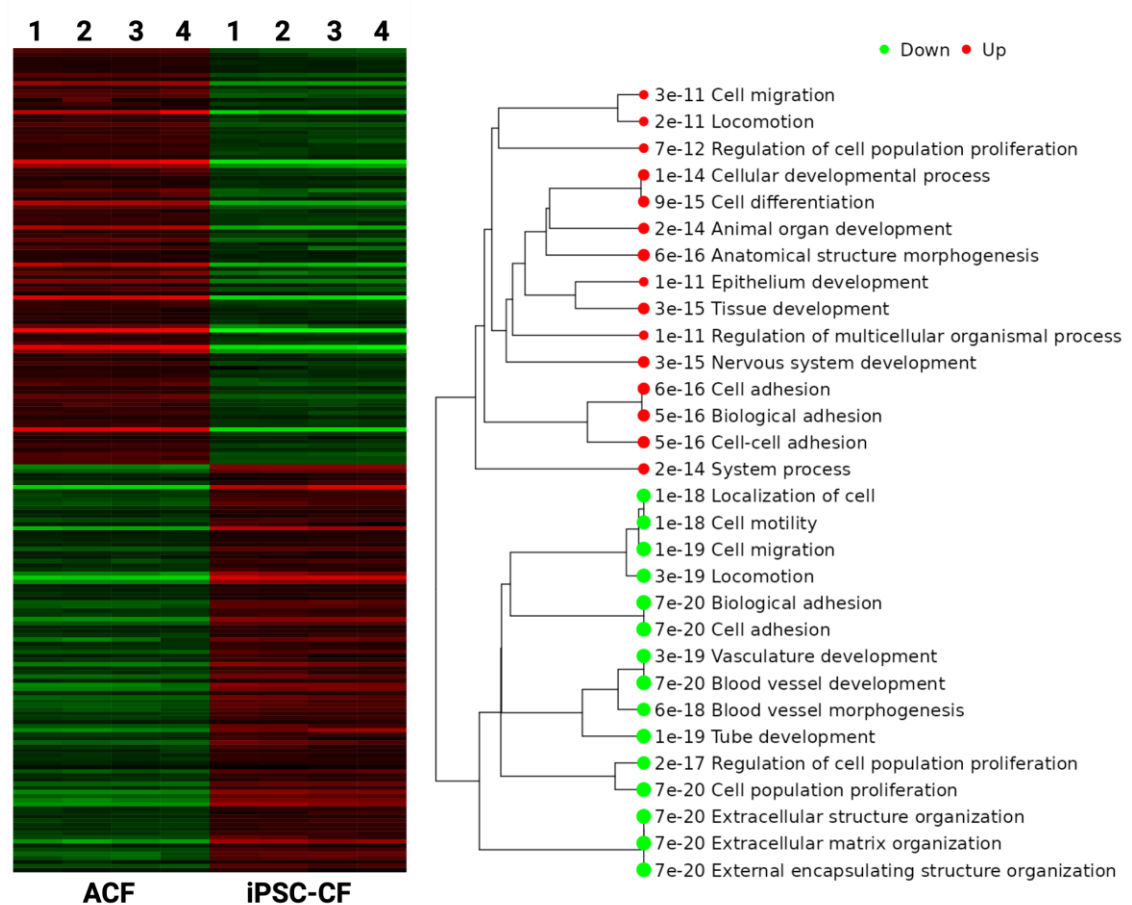


Figure 5.13: Differential Gene Expression (DEG) Analysis of iPSC-CFs and ACFs

Differentially expressed genes in iPSC-CFs and ACFs were identified (FDR < 0.01, Fold Change > 2). **Left:** A heatmap of the upregulated and downregulated genes in ACFs and iPSC-CFs. Relatively higher expression values are shown in red and relatively lower values showed in green. **Right:** An enrichment pathway of upregulated and downregulated processes in iPSC-CFs (Adjusted P-Values are shown on the left of each pathway).

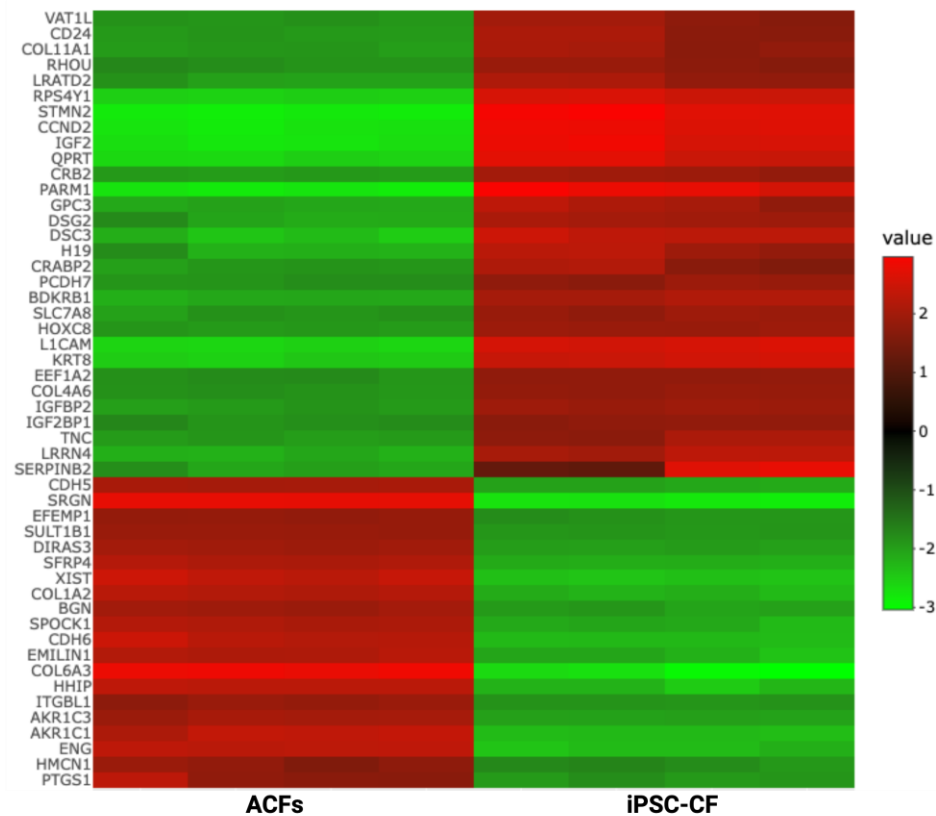


Figure 5.14: Top 50 Differentially Expressed Genes in iPSC-CFs and ACFs

The top 50 differentially expressed genes were identified in the iPSC-CFs and ACFs populations. A normalised expression value is shown on the right, with relatively higher expression values shown in red and relatively lower values shown in green. Genes are listed on the left.

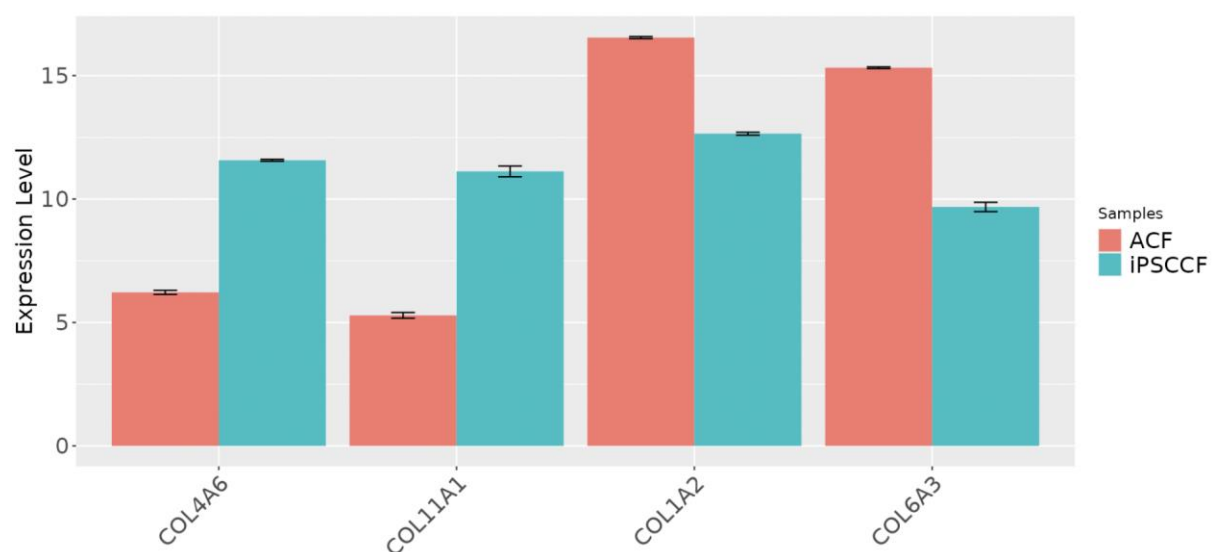


Figure 5.15: Expression of Differentially Expressed Collagen genes in iPSC-CFs and ACFs

Expression of *COL4A6*, *COL11A1*, *COL1A2* and *COL6A3* in ACFs and iPSC-CFs. Expression level is shown on a regularized log scale.

5.3.5.3 Transcriptional Changes of iPSC-CFs following TGF- β 1 Treatment

Differential gene expression analysis of iPSC-CFs with and without 12 hours of TGF- β 1 treatment identified 67 genes significantly downregulated following treatment and 163 upregulated (*Figure 5.16A*). Pathway analysis of the downregulated genes identified no significant common pathways associated with the genes. Conversely, analysis of the upregulated genes identified several significant pathways. A network of the most significantly upregulated pathways following TGF- β 1 treatment are shown in *Figure 5.16B*. The top four most statistically significant pathways determined were extracellular matrix organisation, extracellular structure organisation, external encapsulating structure organisation and anatomical structure morphogenesis. A labelled heatmap of the 50 most up and downregulated genes is shown in *Figure 5.17A*. It identifies an upregulation in pro-fibrotic genes including *TGFBI* and *ADAM19*. Two ion channel subunit genes, *KCNG1* and *KCTD16*, were upregulated in the cells treated with TGF- β 1.

An M-A plot of the significantly upregulated and downregulated genes is shown in *Figure 5.17B*. The most abundantly expressed genes that were significantly upregulated following 12 hours of TGF- β 1 treatment are labelled, and included: *TGFBI*, *SERPINE1*, *FN1*, *COL1A1*, *COL4A2* and *COL5A1*. The most statistically significant genes to demonstrate upregulation are labelled and include *ODAPH*, *BMP6* and *KCTD16*. A STRING analysis of the protein interaction networks of the top 100 upregulated genes is shown in *Figure 5.18*. The analysis identified the proteins encoded by *CTGF*, *TGFBI*, *SPOCK1* and *MXRA5* as showing some of the largest numbers of predicted interactions.

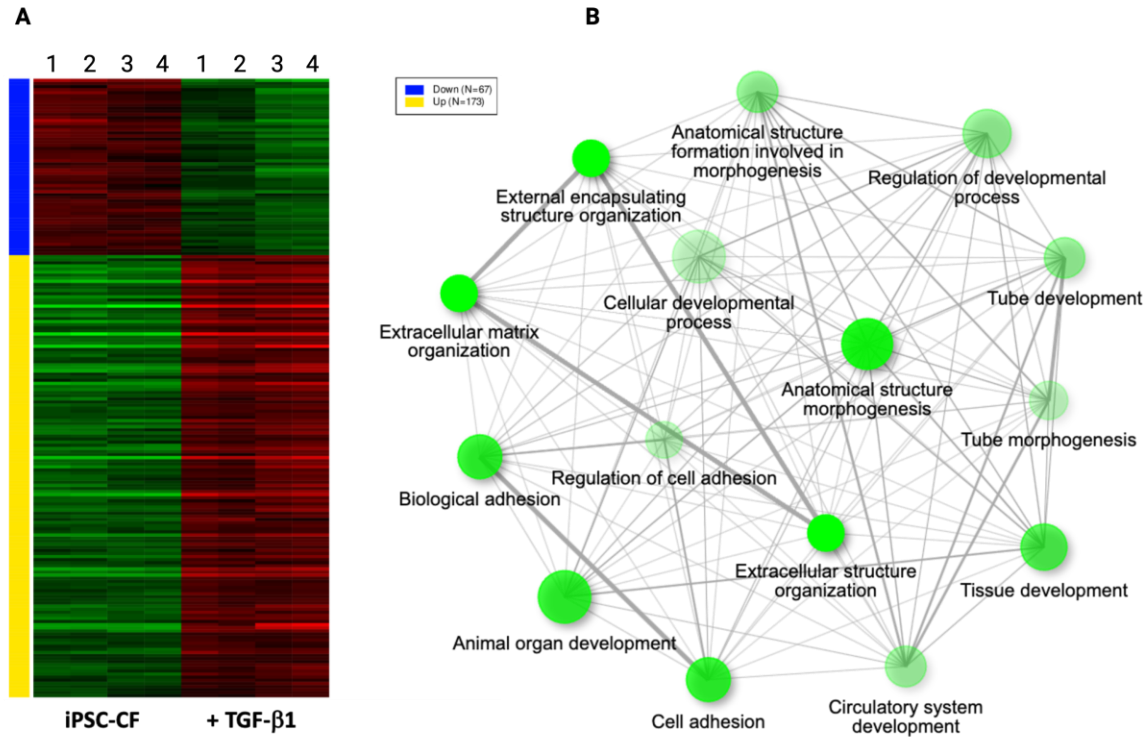


Figure 5.16: Differential Gene Expression (DEG) Analysis of iPSC-CFs with and without TGF-β1 Treatment

Differentially expressed genes in iPSC-CFs following 12 hours of treatment with 10 ng/mL TGF-β1 were identified (FDR < 0.01, Fold Change > 2). **A:** A heatmap of the upregulated and downregulated genes. Relatively higher expression values are shown in red and relatively lower expression values are shown in green. **B:** A network of pathways significantly upregulated in iPSC-CFs following TGF-β1 treatment. Pathways are connected if they have 30% or more genes in common. Larger nodes represent more genes within the pathway. Darker nodes show significantly enriched gene sets.

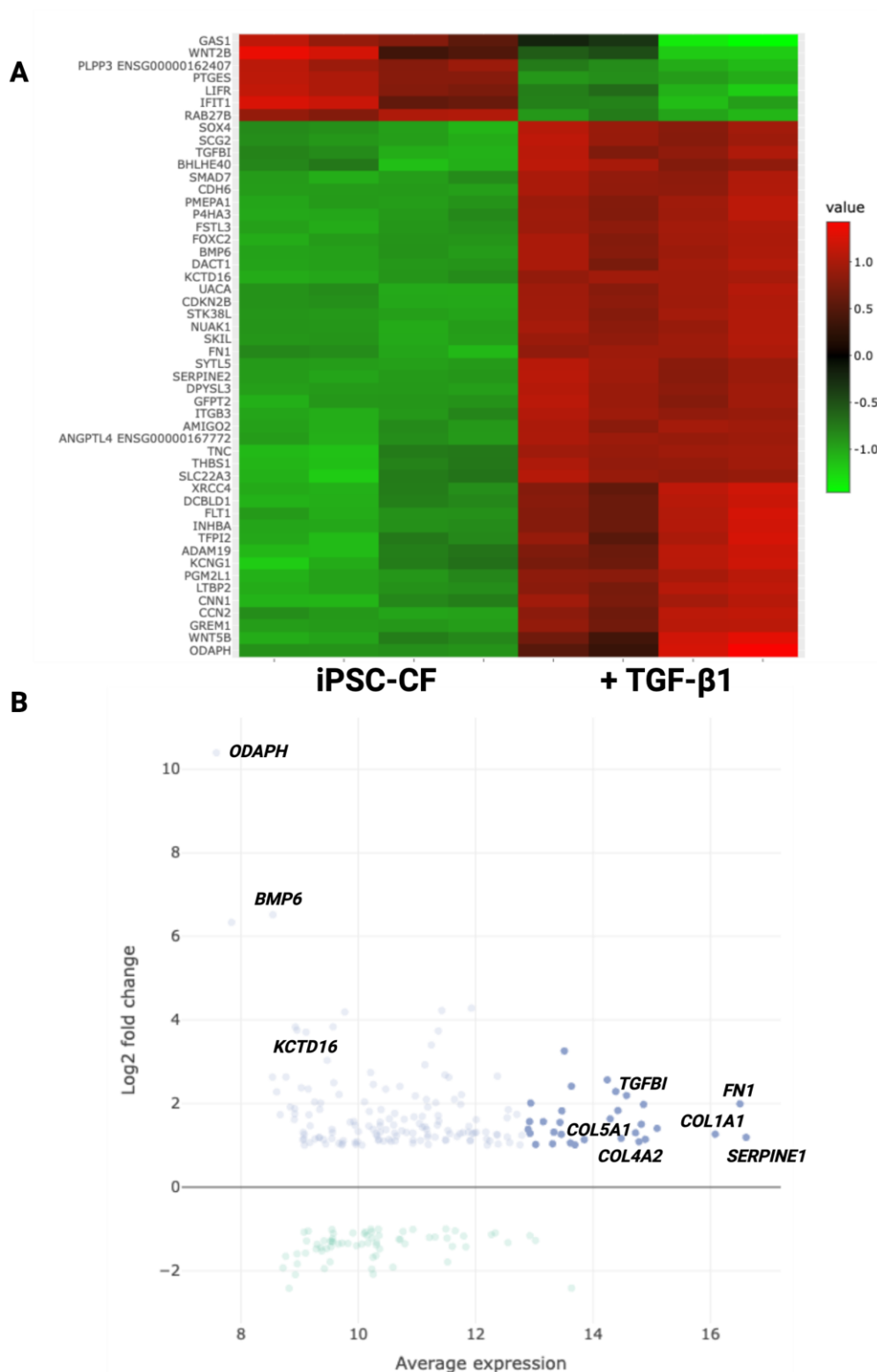


Figure 5.17: Most Variable Genes in iPSC-CFs following TGF-β1 Treatment

A: A heatmap of the top 50 most variable genes in iPSC-CFs following 12 hours of treatment with 10 ng/mL TGF-β1. A key showing the normalised expression value is shown on the right, with relatively higher expression values shown in red and relatively lower values showed in green. **B:** An M-A plot of the significantly upregulated and downregulated genes (FDR < 0.01, Fold Change > 2) following TGF-β1 treatment. Genes of interest have been labelled. Average expression is in a log2 fold change.

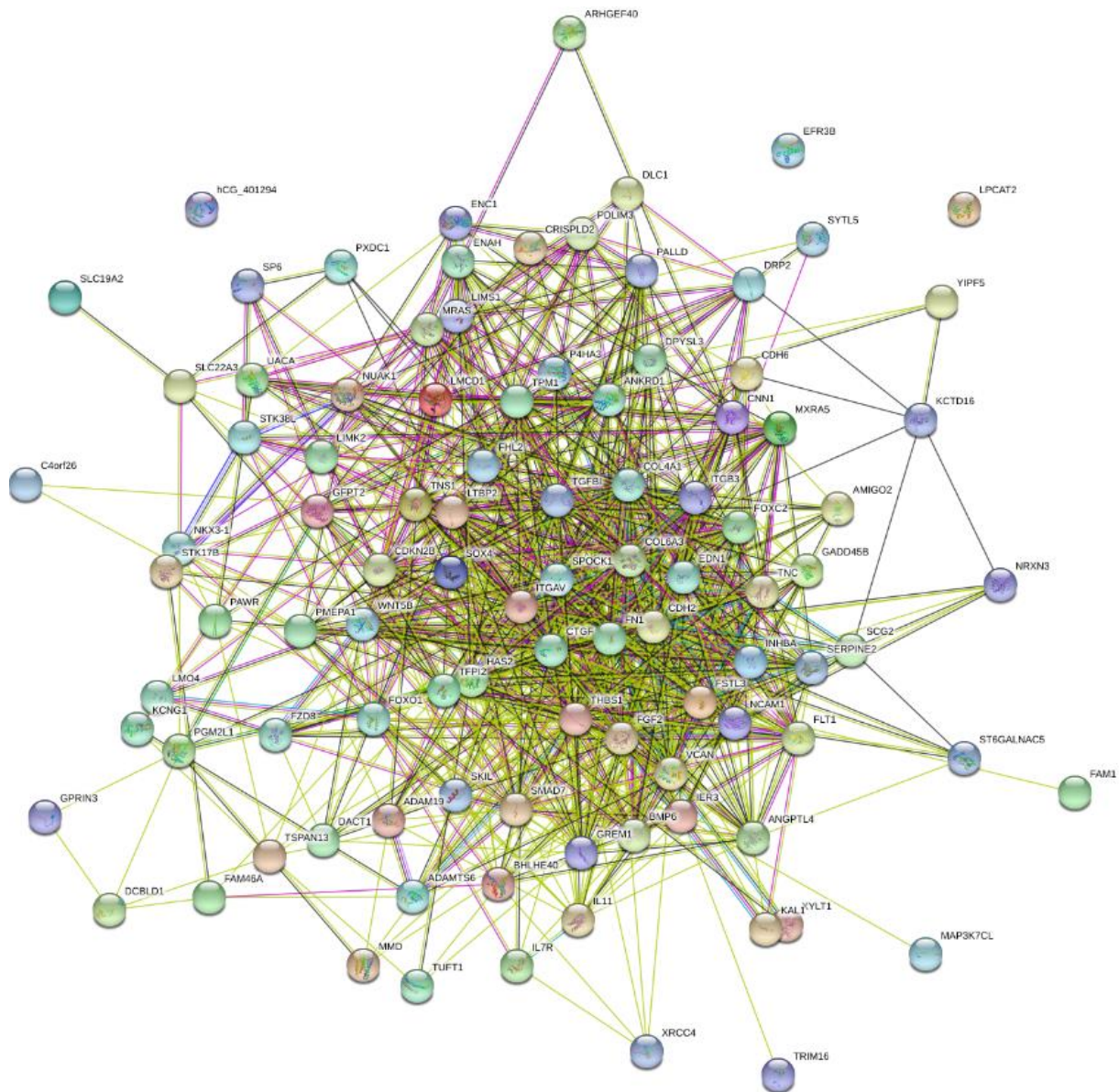


Figure 5.18: Protein Interaction Network of Genes Upregulated in iPSC-CFs following TGF- β 1 Treatment

The top 100 upregulated genes in iPSC-CFs following 12 hours of treatment with 10 ng/mL TGF- β 1 were used in STRING analysis to identify protein interaction networks. The expected number of interactions of 100 randomly chosen proteins would be 311. 849 interactions were identified between the proteins encoded by the upregulated genes in iPSC-CFs.

5.3.5.4 Transcriptional Changes of ACFs following TGF- β 1 Treatment

The ACFs demonstrated a less substantial response to the TGF- β 1 treatment than the iPSC-CFs. This is illustrated in the PCA plot previously shown in *Figure 5.12*. One of the ACF samples treated with TGF- β 1 demonstrated a divergent response in gene expression. This can be more clearly observed in *Figure 5.19*, which shows a PCA plot of the ACF samples with and without TGF- β 1 treatment, as well as a K-means cluster analysis of the top 2000

differentially expressed genes. In the PCA plot, one of the ACF samples treated with TGF- β 1 shows significant variation to the other three samples treated with TGF- β 1. The same sample shows a differential response in the transcription of genes in clusters B and C of *Figure 4.19A/B*. The genes of cluster B are associated with mitosis and cell division. The genes in cluster C are associated with cellular response to chemical stimulus and response to an organic substance.

Overall, the cells treated with TGF- β 1 showed a significant downregulation in genes associated with extracellular matrix organisation and structure (cluster A) and an upregulation in genes associated with cellular response to chemical stimulus. There was no significant change in genes associated with mitosis and cell division. There was a total of 46 DEGs between ACFs treated with and without TGF- β 1. This included a downregulation in 38 genes and an upregulation of eight genes following TGF- β 1 (FDR < 0.01, Fold Change > 2). A heatmap showing the 50 most variable genes is shown in *Figure 5.20*. The ACFs treated with TGF- β 1 demonstrated a significant decrease in the expression of genes including *ADAM19*, *ITGAV*, *MMP2*, *IL6*, *TGFBI* and *COL16A1*. Treatment of ACFs with TGF- β 1 for 12 hours did not cause an upregulation of genes typically associated with cardiac fibrosis. Moreover, many of the genes which were significantly downregulated in TGF- β 1 treated ACFs (*ADAM19*, *ITGAV*, *TGFBI*, *SERPINE1*, *BMP6*), showed upregulation in iPSC-CFs following the same treatment.

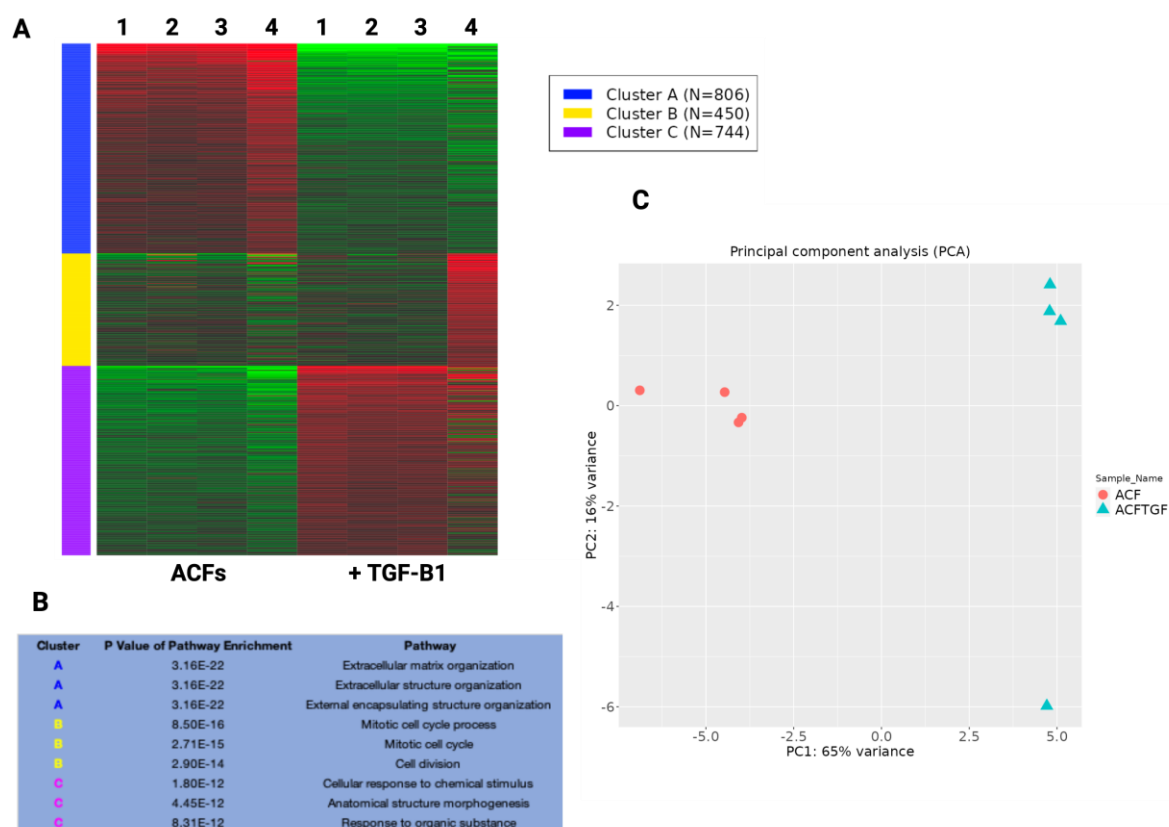


Figure 5.19: Variability in ACFs Expression Profiles Following TGF-β1 Treatment

A: A K-Means clustering analysis was performed on the 2000 most variable genes in ACFs treated with and without TGF-β1 to identify upregulated and downregulated pathways. Sample 4 of the ACFs + **TGF-β1** demonstrated differential gene expression in clusters B and C when compared to the other samples. Higher expression values are shown in red and lower expression values are shown in green. **B:** The 3 most statistically significant pathways associated with each group of genes is shown. **C:** A PCA plot showing the first and second principal components used to separate the transcriptional profiles of ACFs with and without TGF-β1 treatment.

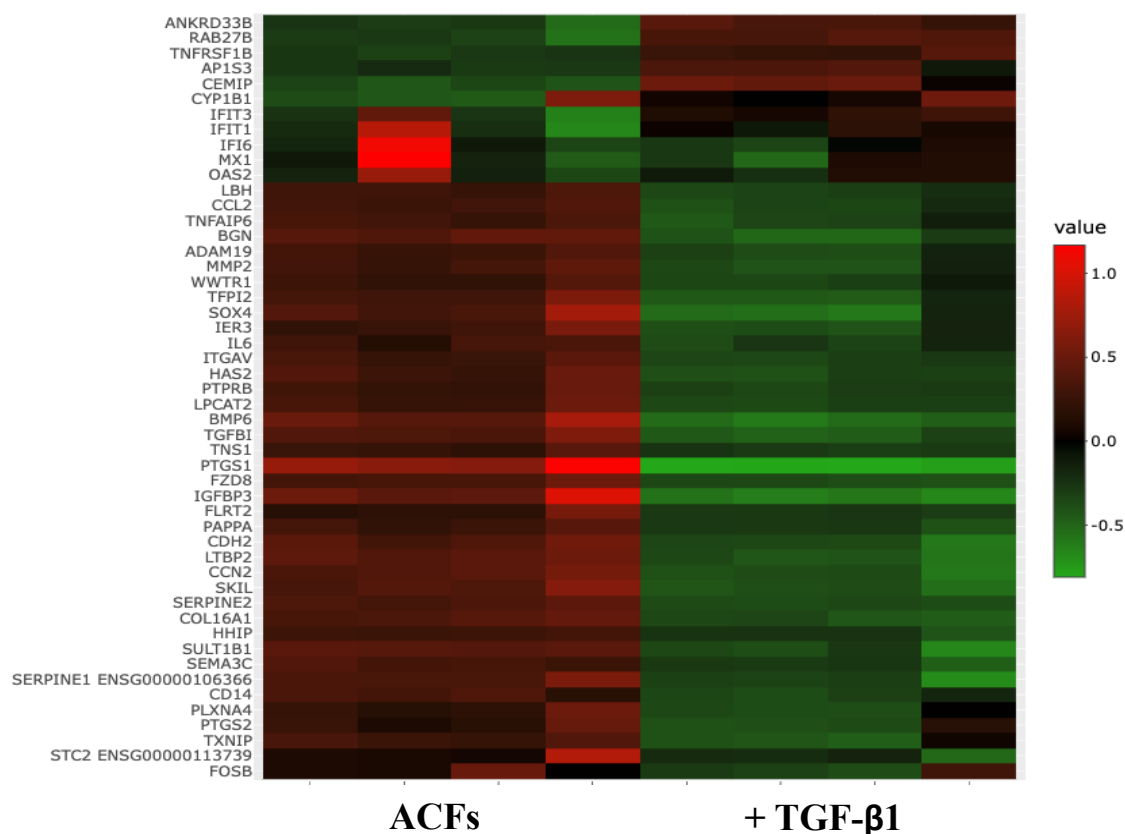


Figure 5.20: Most Variable Genes in ACFs following TGF-β1 Treatment

A heatmap of the top 50 most variable genes in ACFs following 12 hours of treatment with 10 ng/mL TGF-β1. Higher expression values are shown in red and lower values showed in green.

5.3.6 Quantitative Proteomics of iPSC-CFs and ACFs

5.3.6.1 Experimental Setup

Quantitative proteomics was performed to assess the effect TGF-β1 treatment had on cardiac fibroblast activation and to detect changes in ion channel expression that may be integral to the myofibroblast phenotype. The iPSC-CFs and ACFs were treated with either 10 ng/mL TGF-β1 and 10 ng/mL FGF2 (Activation Media) or 10 μM TGF-β1 inhibitor (SB431542) and 10 ng/mL FGF2 (Quiescence Media) for 48 hours. 5 biological replicates, differing by at least 1 passage, were used for each condition (Cell type, +/- TGF-β1). The cells were spun down and pellets were harvested. A 48-hour treatment period was used to allow adequate time for the cardiac fibroblasts to become active and for changes in protein levels to manifest.

Protein samples were processed and labelled as described in 5.2.2. The labelled peptide samples of the iPSC-CFs (5 x no TGF- β 1, 5 x + TGF- β 1) and ACFs (5 x no TGF- β 1, 5 x + TGF- β 1) were analysed on separate mass spectrometry runs. This meant that the abundance of proteins identified in the iPSC-CF samples could not be directly compared to those from the ACF samples. Only samples with and without TGF- β 1 treatment could be compared e.g. iPSC-CF vs iPSC + TGF- β 1 and ACF vs ACF + TGF- β 1.

5.3.6.2 Protein Expression Changes in iPSC-CFs and ACFs Following TGF- β 1 Treatment

Differentially expressed proteins (DEPs) were identified between iPSC-CFs with and without TGF- β 1 treatment (Adj P Value < 0.05, Fold Change > 1.5). A total number of 35 DEPs (15 up, 20 down) were determined for the iPSC-CF samples and are listed in *Table 5.5*. Many of the significantly upregulated proteins including dihydropyrimidinase-related protein 3, transgelin, calponin, caldesmon and tropomyosin are involved in cytoskeletal/actin reorganisation and myofibroblast differentiation. There was an approximately 2-fold increase in the extracellular matrix protein fibronectin in the TGF- β 1 treated iPSC-CFs. Significantly downregulated proteins included intercellular adhesion molecule 1, neprilysin, CD9 antigen and the sodium-coupled neutral amino acid transporter 2. No ion channel subunits proteins were identified as being significantly up or downregulated. A volcano plot showing the changes in protein expression following TGF- β 1 treatment is shown in *Figure 5.21*. The proteins that show the most statistically significant change in expression are identified and labelled, including actin cytoplasmic 1. Only two DEPs (Adj P Value < 0.05, Fold Change > 1.5) were identified between the ACFs with and without TGF- β 1 treatment: There was a significant upregulation of importin subunit alpha-1 and a downregulation of superoxide dismutase. Neither of them were found to be DEPs in the iPSC-CFs.

Table 5.5: DEPs in iPSC-CFs with and without TGF- β 1 Treatment

Description	Gene Symbol	Adj P Value	Fold Change
Dihydropyrimidinase-related protein 3	DPYSL3	0.00031412	2.664
Transgelin	TAGLN	0.00065426	2.508
Calponin-1	CNN1	1.62E-06	2.361
Fibronectin	FN1	3.83E-05	1.954
Tropomyosin beta chain	TPM2	0.00065426	1.89
Tropomyosin alpha-1	TPM1	1.62E-06	1.882
Caldesmon	CALD1	8.45E-05	1.846
Four and a half LIM domains protein 2	FHL2	0.00514141	1.807
Glia-derived nexin	SERPINE2	0.00120158	1.789
Importin-4	IPO4	0.00376871	1.709
Myosin regulatory light polypeptide 9	MYL9	0.00278455	1.693
Fructose-2,6-bisphosphatase	TIGAR	0.00376871	1.615
Calponin-2	CNN2	0.00087928	1.605
Integrin alpha-V	ITGAV	0.00118903	1.574
5'-AMP-activated protein kinase subunit gamma-1	PRKAG1	0.01863599	1.571
Superoxide dismutase	SOD2	0.00141201	0.669
Histone H2A type 2-A	HIST2H2AA3	0.00318338	0.662
Ras-related protein Rab-7a	RAB7A	0.00358139	0.659
ATP synthase-coupling factor 6	ATP5PF	0.00402433	0.656
Lamin-B1	LMNB1	0.0016363	0.655
MARCKS-related protein	MARCKSL1	0.00226421	0.655
Basigin	BSG	0.00921196	0.654
Ferritin light chain	FTL	0.00142377	0.642
Microsomal glutathione S-transferase 1	MGST1	0.00098141	0.631
10 kDa heat shock protein, mitochondrial	HSPE1	0.00150783	0.624
Lanosterol 14-alpha demethylase	CYP51A1	0.00068105	0.618
Interferon-induced transmembrane protein 3	IFITM3	0.00449986	0.583
Cytochrome b5 type B	CYB5B	0.0026171	0.576
Ferritin heavy chain	FTH1	0.00118903	0.559
CD44 antigen	CD44	0.00062078	0.558
Neprilysin	MME	0.00010743	0.534
Intercellular adhesion molecule 1	ICAM1	3.95E-05	0.508
Erythrocyte band 7 integral membrane protein	STOM	0.00107313	0.478
CD9 antigen	CD9	0.00117778	0.478
Sodium-coupled neutral amino acid transporter 2	SLC38A2	0.00046243	0.465

Protein Expression of iPSC-CF

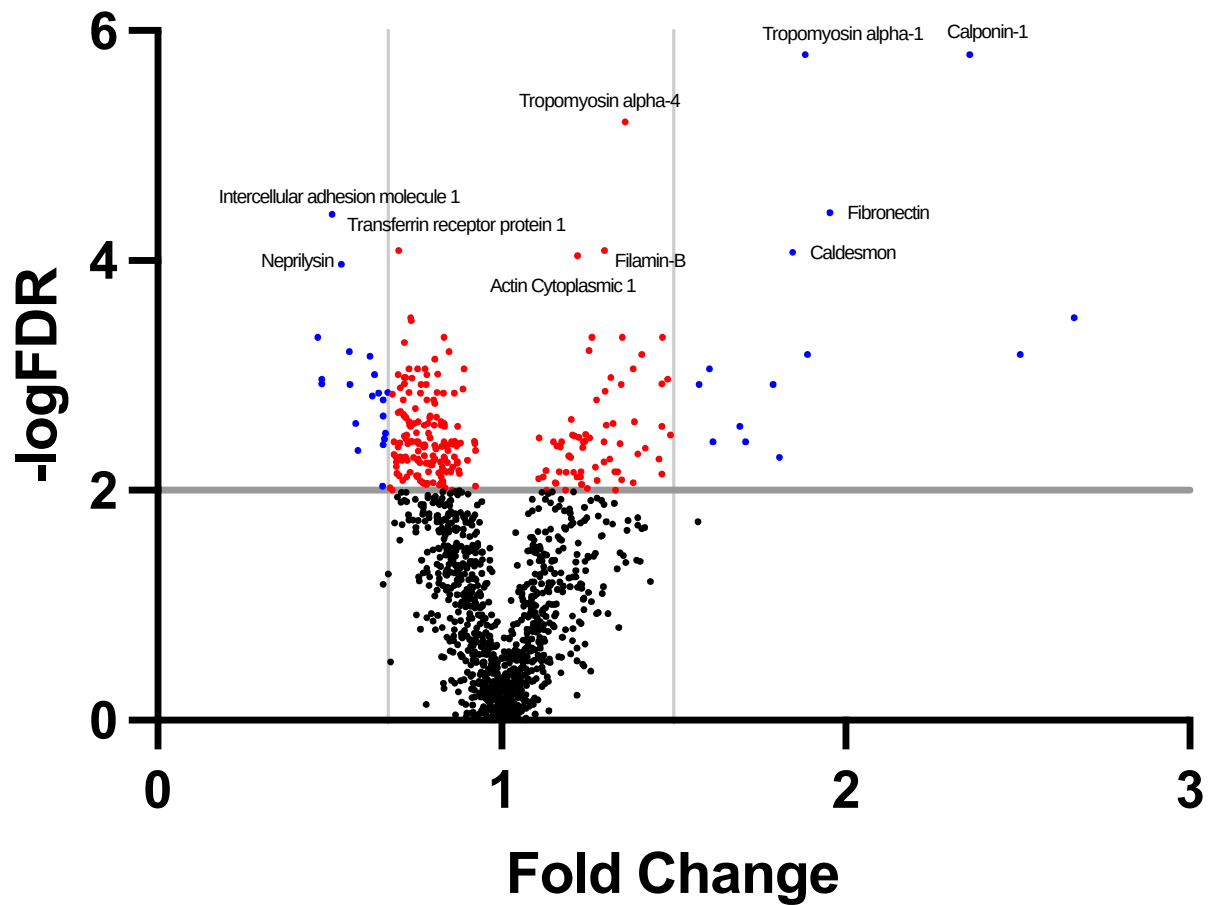


Figure 5.21: Changes in Protein Expression in iPSC-CFs following TGF- β 1 Treatment

A volcano plot showing the proteins identified in samples of iPSC-CFs with and without 48 hours of 10 ng/mL TGF- β 1 treatment. Fold Change and -logFDR are respectively shown on the x and y axis. Proteins identified with a -logFDR > 2 (Adj P Value < 0.05) are shown in red. DEPs with a Adj P Value < 0.05 and a fold change greater than 1.5 are shown in blue. The proteins identified with the largest -logFDR are labelled, regardless of fold change.

5.4 Discussion

5.4.1 Cardiac Fibroblast Activation

The cardiac fibroblasts demonstrated varying levels of activation following treatment with 10 ng/mL TGF- β 1 for 72 hours. The iPSC-CFs demonstrated the greatest increase in *ACTA2* and α SMA expression following treatment with TGF- β 1 consistent with previous studies indicating greater plasticity than their primary counterparts (Moita et al., 2022). An increase in collagen 1 expression was observed in the TGF- β 1 treated iPSC-CFs and was consistent with increased expression of the *COL1A1* gene. The VCFs demonstrated greater expression of collagen 1 following TGF- β 1 treatment. The cells showed basal expression of *ACTA2* and α SMA, which substantially increased following TGF- β 1 treatment. The VCFs were kindly gifted to the lab at passage 9. Cardiac fibroblasts can become activated over prolonged time in culture (Petrov et al., 2022). Therefore, the basal expression of α SMA and collagen 1 in the VCFs was likely because the cells were already partially activated. The ACFs stained positive for collagen 1 prior to TGF- β 1 treatment and demonstrated a minimal increase in its expression following treatment, despite showing a 3-fold increase in gene expression. The ACFs demonstrated a marginal increase in expression of *ACTA2* and α SMA following TGF- β 1 treatment. Overall, the iPSC-CFs and VCFs showed increases in cardiac fibroblast activation following treatment with TGF- β 1 for 72 hours. The ACFs showed limited plasticity for collagen 1 and almost no expression of α SMA when treated with TGF- β 1. Assaying expression of other activation markers including periostin, osteopontin and paxillin would have been beneficial in confirming the activation plasticity of the cardiac fibroblasts (Tarbit et al., 2019).

5.4.2 Gene Expression of Ion Channel Subunits in iPSC-CFs, VCFs and ACFs

The expression of ion channel genes identified as being important in cardiac fibroblast activation were assessed in iPSC-CFs, VCFs and ACFs prior to and following the addition of TGF- β 1.

5.4.2.1 TRP Gene Expression

Transient receptor proteins have previously been shown to be strongly expressed in the heart and to play a role in cation (predominantly Ca^{2+}) influx during cardiac fibroblast activation (Freichel et al., 2017) (Gwanyanya and Mubagwa, 2022). Influx of Ca^{2+} causes the activation of calcineurin which subsequently facilitates the translocation of NFAT to the nucleus and expression of pro-fibrotic genes including *ACTA2* and *COL3A1* (Herum et al., 2013). The iPSC-CFs and VCFs showed either a downregulation or no significant difference in expression of the TRP genes following TGF- β 1 treatment. This was unexpected and in contrast with previous studies demonstrating increases in expression following TGF- β 1 treatment (Ahn et al., 2020) (Du et al., 2010). ACFs subsequently showed no difference in expression of *TRPM7* and *TRPC4* expression following TGF- β 1 treatment, but a significant increase in *TRPV4* expression.

There are a number of differences in culture conditions used, which may explain the reduced plasticity in *TRP* gene expression. In both of the previous studies mentioned, a serum starvation was performed prior to treatment of the cells with lower concentrations of TGF- β 1. Furthermore, the cells cultured in this study were cultured on a geltrex substrate as opposed to uncoated plastic. Finally, FGF2 which was supplemented into both the quiescent and activation media in this study, has been shown to regulate the activity of TRP channels and may therefore have blunted the response (Van den Eynde et al., 2021).

5.4.2.2 Voltage-gated Ion Channel Expression

The iPSC-CFs and VCFs respectively demonstrated a significant increase and decrease in *SCN5A* expression following treatment with TGF- β 1. Previous studies on atrial cardiac fibroblasts have demonstrated an increase in *SCN5A* gene expression and inward sodium current activity following cardiac fibroblast activation (Chatelier et al., 2012). However, the functional relevance of an increase in inward sodium current in cardiac fibroblasts is still unclear. Furthermore, an increase in expression of *SCN5A* is not necessarily indicative of increased current activity.

The iPSC-CFs and VCFs demonstrated similar changes in gene expression of *CACNA1C*, *KCND2*, *KCNB1*, *KCNJ2* and *KCNMA1*. A significant increase in expression of *CACNA1C*, which encodes a subunit of the L-type calcium channel, was observed in the VCFs. L-type calcium channel activity has been previously measured in cardiac myofibroblasts (Bae et al., 2020). However, the exact role they play in cardiac fibroblast activation and myofibroblast electrophysiology is controversial and enigmatic, with other studies hesitant on their functional relevance (Chen et al., 2010). Increases in L-type calcium channel activity following cardiac fibroblast activation may contribute to the depolarisation of the myofibroblast membrane potential.

As previously mentioned, the *KCNB1* gene encodes a subunit of the K_v2.1 potassium channel, which has a variety of roles including regulating Ca²⁺ channel function and membrane potential (O'Dwyer et al., 2020). A significant downregulation of the gene was observed in both iPSC-CFs and VCFs following TGF- β 1. A decrease in K_v2.1 expression and function could cause a decrease in K⁺ efflux and may subsequently contribute to the depolarisation of the myofibroblast membrane potential. Cardiac fibroblasts have previously been shown to express K_v2.1, however it has not been functionally characterised in cardiac fibroblasts. ((Li et al., 2009). Future experiments using voltage clamp to investigate the

functional role this ion channel plays on cardiac fibroblast electrophysiology may be beneficial.

The VCFs demonstrated a significant increase in expression of *KCNJ2*, which encodes a subunit of the inward rectifying potassium current (K_{ir}). Increased expression and activity of K_{ir} has been demonstrated in the cardiac fibroblasts of dogs with congestive heart failure (Qi et al., 2015). The upregulation was linked to a depolarisation of the membrane potential and increased Ca^{2+} influx.

A significant decrease in *KCND2* (I_{to}) expression was observed in both the iPSC-CFs and VCFs following treatment with TGF- β 1 and is consistent with previous studies (Kaur et al., 2013). The transient outward potassium current (I_{to}) is expressed in cardiac fibroblasts; however, the functional consequences of downregulation are unclear (Li et al., 2009).

Robust expression of *KCNMA1*, which encodes a calcium sensitive potassium current known as a BK channel, was observed in the iPSC-CFs, VCFs and ACFs. All three cardiac fibroblast cell lines showed no significant change in *KCNMA1* expression following TGF- β 1 treatment. Nevertheless, it has been shown that the activity of BK channels can be upregulated by the increased activity of piezo channels. Therefore, high expression of BK channels may be of functional relevance despite unchanged expression.

Functional experiments are needed to validate whether changes observed in gene expression cause a change in ion channel density. Furthermore, it is currently unclear to what degree the membrane potential of cardiac fibroblasts changes during signal propagation through the heart and whether many of the voltage gated ion channels discussed reach the membrane potential required to open when *in situ*.

5.4.2.3 Piezo Channels

Stretch activated piezo channels are predominantly permeable to Ca^{2+} , and when activated cause an increase in intracellular Ca^{2+} concentrations. Increased activity of piezo channels can cause activation of BK channels, which have shown to increase cardiac fibroblast proliferation and promote cardiac remodelling (Jakob et al., 2021) (Sheng et al., 2013). Furthermore, the increased activity of Piezo1 and the concomitant increase in intracellular Ca^{2+} has been linked to calcineurin mediated NFAT translocation and induction of pro-fibrotic genes (Zhang et al., 2021).

The iPSC-CFs and VCFs demonstrated no significant change in expression of *PIEZO1* following TGF- β 1 treatment. The iPSC-CFs showed a significant increase in expression of *PIEZO2* following TGF- β 1 treatment, however the VCFs showed a contrasting decrease. It is unclear why the cardiac fibroblast cell lines demonstrated variable responses in *PIEZO* expression following TGF- β 1 treatment. Culture of the cells on stiff plastic may disrupt the regulation of these predominantly mechanosensitive channels (Gilles et al., 2020).

Overall, the iPSC-CFs and VCFs demonstrated similar changes in expression of voltage gated ion channel subunit genes following TGF- β 1 treatment. All three cardiac fibroblast cell lines responded differently in terms of mechanosensitive channel expression following TGF- β 1 treatment. It is likely that the long-term culture of cardiac fibroblasts on stiff plastic cell culture plates causes disruption to both the regulation and expression of mechanosensitive ion channels. Future experiments should utilise soft substrates with more physiological stiffnesses (Xie et al., 2014).

5.4.3 RNA-Seq of iPSC-CFs and ACFs

5.4.3.1 Transcriptional Differences between iPSC-CFs and ACFs

The iPSC-CFs and ACFs demonstrated significant differences in their transcriptomes at baseline, with over 1500 DEGs between the cell types. The iPSC-CFs showed significantly greater expression of genes associated with anatomical structure morphogenesis and cell differentiation such as *STMN2* and *L1CAM*. *STMN2* encodes the Stathmin-2 protein which can regulate microtubule disassembly and promote TGF- β signalling and epithelial to mesenchymal transition (Zhong et al., 2021). Stathmin-2 has been shown to help regulate α SMA synthesis in vascular smooth muscle cells (Ke et al., 2022). The iPSC-CFs demonstrated high expression of *STMN2*, whilst the ACFs demonstrated no detectable expression of the gene. This dichotomy may partially explain the paucity of α SMA expression in the ACFs following TGF- β 1 treatment. The *L1CAM* gene encodes a cell adhesion protein predominantly expressed on the surface of neurones (Linneberg et al., 2019). The high expression of *L1CAM* in iPSC-CFs may occur as a transcriptional remnant from stem cell differentiation.

The iPSC-CFs were downregulated for the following gene pathways: blood cell development, cell adhesion, cell population proliferation, biological adhesion and extracellular matrix organisation. Overall, the ACFs showed significantly higher expression of extracellular matrix encoding genes including *COL6A3* and *COL1A2*. The substantially higher expression of *COL1A2* in ACFs may explain the basal expression of type 1 collagen observed by immunofluorescence in 5.3.2.1.

5.4.3.2 RNA-Seq of iPSC-CFs with and without TGF- β 1 Treatment

The iPSC-CFs demonstrated an increase in expression of genes associated with myofibroblast differentiation following TGF- β 1 treatment, including *TGFBI* and *ADAM19*. Indeed, these two genes have previously been identified using bioinformatic analysis as the most important mRNAs during fibrosis in Gao et al., (2021), with knockdown of either gene abrogating

expression of *ACTA2* and *COL1A1*. There was a substantial increase in expression of extracellular matrix genes including *FN1*, *COL1A1*, *COL4A2* and *COL5A1*, suggesting activation of the iPSC-CFs. Treatment with TGF- β 1 caused a significant increase in the expression of two ion channel subunit genes *KCNG1* and *KCTD16* following the 12 hours of TGF- β 1 treatment. *KCNG1* encodes an ion channel subunit (K_v6.1) that interacts and modulates activity of the membrane potential regulating channel K_v2.1 (*KCNB1*), whose expression was previously shown to be downregulated during fibroblast activation (Kramer et al., 1998). Altered activity of K_v2.1 is likely important in the depolarisation of the membrane potential of cardiac fibroblasts during activation. Further functional experiments exploring the role *KCNG1* (K_v6.1) has on cardiac fibroblast activation may help elucidate this interaction. STRING analysis implicated the proteins encoded by *CTGF*, *TGFBI*, *SPOCK1* and *MXRA5* as having some of the largest numbers of predicted interactions during treatment of iPSC-CFs with TGF- β 1. Many of these genes are known to be pro-fibrotic. For example, *CTGF* encodes connective tissue growth factor, which plays a fundamental role in TGF- β 1 dependent cardiac remodelling and fibrosis (Dorn et al., 2018).

5.4.3.3 RNA-Seq of ACFs with and without TGF- β 1 Treatment

The ACFs demonstrated significantly less changes than the iPSC-CFs in gene expression following TGF- β 1 treatment with only 46 DEGs identified. This is partially explained by the significantly greater variation between the biological replicates of ACFs, likely caused by iterative transdifferentiation of the cells during time in culture. Overall, however, the cells showed minimal transcriptional response to 12 hours of TGF- β 1 treatment. The reason for this unresponsiveness is unclear. One explanation consistent with the high expression of ECM genes in the ACFs would be that the cells were already chronically activated and thus unable to activate further. However, the lack of α SMA expression in 5.3.2.1 would

contradict this and indicate a more quiescent phenotype. Even more alarming was that treatment of the cells with TGF- β 1 caused the downregulation of known pro-fibrotic genes including *COL16A1*, *TGFBI*, *IL6*, *ITGAV* and *MMP2*. As the cells were purchased from a supplier, instead of derived from fresh tissue samples, little information is known on their origin, disease status and previous handling. Positive expression of collagen type 1 and negative expression of factor VIII-related antigen were the only markers used to test cardiac fibroblast status. Therefore, it is possible that the cells provided were not representative of healthy atrial cardiac fibroblasts. Future experiments should endeavour where possible to use freshly isolated primary cardiac fibroblasts.

5.4.4 Protein Expression in iPSC-CFs and ACFs

The iPSC-CFs demonstrated significant increases in the expression of proteins that interact and remodel the actin cytoskeleton including transgelin, calponin, caldesmon and tropomyosin. This is consistent with the expectant increase of α SMA expression in myofibroblasts. There was a statistically significant increase in expression of cytoplasmic actin in the iPSC-CFs treated with TGF- β , however the difference in expression fell short of the 1.5-fold change threshold. A significant increase in expression of the extracellular matrix protein fibronectin indicates that the fibroblasts were activated. There was a downregulation of intercellular adhesion molecule 1, neprilysin, CD9 antigen and the sodium-coupled neutral amino acid transporter 2. Decreasing the activity of the endopeptidase protein neprilysin has been reported as a potential strategy for mitigating maladaptive cardiac remodelling (Burke et al., 2019). It is possible that the downregulation of neprilysin in TGF- β 1 treated iPSC-CFs is an adaptive response to overactivation of the cells. Similar to the transcriptomics data discussed in 5.3.5.4 the ACFs showed only minimal changes in protein expression following TGF- β 1 treatment.

5.4.5 Conclusion

The iPSC-CFs and VCFs demonstrated significant plasticity in regard to TGF- β 1 activation and similar responses in the gene expression of predominantly voltage gated ion channel subunits following TGF- β 1 treatment. Treatment with TGF- β 1 did not cause a coordinated increase in the expression of stretch activated ion channel subunit genes in iPSC-CF, VCFs or ACFs. This is likely due to the cells being cultured on stiff plastic that disrupts the regulation of cellular mechanotransduction. Treatment with TGF- β 1 caused consistent changes in the expression of voltage gated ion channel subunit genes including *KCNB1*, *KCNJ2* and *CACNA1C* in iPSC-CFs and VCFs. The functional consequences of such changes in gene expression could be validated using a single cell voltage clamp setup. RNA-seq analysis revealed severe differences in the transcriptomes of iPSC-CFs and ACFs. The iPSC-CFs demonstrated increased pro-fibrotic gene expression following TGF- β 1 treatment, whilst the ACFs were transcriptionally inert to treatment. The ACFs should not have been used as a comparison to iPSC-CFs in RNA-seq and quantitative proteomics as they did not show plasticity in cardiac fibroblast activation. The VCFs would have made a more suitable primary control. The unresponsiveness of the ACFs used in this study to TGF- β 1 treatment made ascertaining the suitability of iPSC-CFs as a replacement of primary cardiac fibroblasts difficult. Future experiments should endeavour to use cardiac fibroblasts freshly isolated/derived from healthy human cardiac samples. Difficulty in obtaining a physiologically relevant primary cardiac fibroblast cell line demonstrates the need for better models to study cardiac fibrosis.

Chapter 6 Final Discussion

6.1 Summary of Results

The unifying theme of the four research chapters outlined above is the utility of iPSC derived models in cardiovascular disease research. Cardiovascular disease has and will likely continue to be a major cause of mortality worldwide. 19 million mortalities were attributed globally to cardiovascular disease in 2020, which is an increase of approximately 20 % since 2010 (Tsao et al., 2022). Atrial fibrillation (AF) is present in approximately 3 % of adults worldwide, making it the most common cardiac arrhythmia. It confers a significant health risk, with 5 % of patients diagnosed with AF succumbing to cardiovascular complications (predominantly SCD and stroke) each year (Gomez-Outes et al., 2017). The development of novel therapeutics for rhythm and rate control in AF has broadly ground to a halt, with the last drug (Dronedarone) receiving approval by the FDA back in 2009 (Ang et al., 2020). This is mainly thought to be due to a ‘one size fits all’ strategy to treatment that neglects the underlying mechanisms of the disease. Drivers of AF can substantially differ between patients and are heavily influenced by genetic risk factors. Development of novel and effective drugs for AF is likely contingent on the use of physiological models that are easily genetically manipulated.

Small animal models have proven useful in exploring the basic mechanisms behind heart disease but have been hampered by significant species dependent differences in anatomy and physiology. Large animal models are capable of recapitulating much of the complexities of human heart disease, however there is neither the resources nor appetite for their widespread use in cardiovascular disease modelling. Access to primary human cardiac tissue is sporadic, and when available there is often a scarcity of healthy tissue controls. iPSCs have emerged as a renewable and genetically malleable source of human cardiac tissue but have been

hampered by two significant problems. The first problem is that cardiac cells, notably cardiomyocytes differentiated from iPSCs, show phenotypic immaturity and are often incapable of recapitulating age dependent mechanisms of disease. The second problem is that many heart diseases are complex and often involve dysfunction in and between multiple cell types. Monolayers of iPSC-CMs cannot emulate the 3D cell to cell and cell to matrix interactions present in the myocardium. This thesis attempted to generate and validate iPSC derived cardiac models for use in disease modelling. Particular attention was given to exploring the pathogenesis of a titin truncating variant which predisposes individuals to early onset AF and DCM.

The principal aims of this project were to:

1. Generate atrial iPSC-CMs that could act as a model to investigate mechanisms behind atrial fibrillation (Chapter 2)
2. Generate an iPSC derived 3D cardiac model that can be used to explore structural remodelling *in-vitro* (Chapter 3)
3. Interrogate the electrical and structural mechanisms associated with TTNtv associated AF (Chapter 4)
4. Investigate the changes that occur in intracellular electrophysiology during cardiac fibroblast activation (Chapter 5)

6.1.1 Chapter 2: Generation and Validation of a Human Atrial iPSC-CM Model

Chapter 2 described the generation and validation of atrial iPSC-CMs from KOLFC2 iPSCs. Work in the laboratory, prior to the start of the project had focused on the ramifications pathogenic variants have in ventricular iPSC-CMs, with no previous work having been performed modelling atrial disease. Optimising and validating the differentiation of atrial

iPSC-CMs was therefore necessary prior to assessing the effect a pathogenic variant had on AF. A summary of the results delineated in this chapter are shown in *Figure 6.1*.

Manipulation of the retinoid signalling pathway for the generation of atrial like iPSC-CMs is well established (Gassanov et al., 2008). Addition of 1.0 μ M retinoic acid proved useful in generating atrial iPSC-CMs and is consistent with previous studies that overwhelmingly favour the use of this concentration (Wiesinger et al., 2021). The atrial iPSC-CMs derived demonstrated an upregulation in atrial specific structural and electrophysiological genes/proteins (*MYH6*, *MYL7*, *NPPA*, *KCNJ3* and *MLC-2a*) and the downregulation of ventricular markers (*MYL2* and *MYH7*). Perhaps more pertinent to investigating the pathophysiology of AF, the action potentials derived from the cells showed atrial like characteristics relative to those from the ventricular iPSC-CMs (slowed upstroke velocities, shortened APDs, more positive diastolic membrane potentials, atrial like AP morphology). In contrast to previous studies (Ahmad et al., 2023), the gene expression of *KCNA5* ($K_v1.5$) and *KCNJ5* (*GIRK4*) was not upregulated in the atrial iPSC-CMs. It is unclear as to why the cells did not demonstrate an increase in expression of these genes. Batch to batch variability is a known issue with iPSC-CMs and likely contributed towards the unexpected results. Differences in the iPSC line used may also have played a part. Future experiments should aim to reduce batch-to-batch variability by setting quality control thresholds. These could include a minimum percentage of iPSC-CMs expressing a cardiomyocyte marker such as troponin T (Mannhardt et al., 2020).

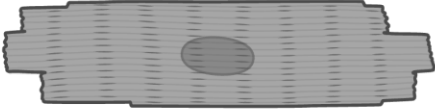
Ventricular	Atrial
	
Genetic Markers	
<i>MYH7, MYL2, KCNA4</i>	<i>MYH6, MYL7, NPPA, KCNJ3</i>
Structural Markers	
MLC-2v	MLC-2a
Electrophysiological Markers	
Prolonged APD Hyperpolarised MDP Increased Upstroke Velocities	Shortened APD, Depolarised MDP Decreased Upstroke Velocities

Figure 6.1: Summary of Chapter 2

A summary of the results detailed in Chapter 2. The ventricular iPSC-CMs demonstrated increased expression of *MYH7*, *MYL2* and *KCNA4*. The cells predominantly expressed the ventricular isoform of the myosin light chain. The cells demonstrated prolonged APDs, increased upstroke velocities and hyperpolarisation of the diastolic membrane potential. The atrial iPSC-CMs demonstrated increased expression of *MYH6*, *MYL7*, *NPPA* and *KCNJ3*. The cells predominantly expressed the atrial isoform of the myosin light chain. The cells demonstrated shortened APDs, decreased upstroke velocities and a depolarised diastolic membrane potential.

6.1.1.1 Limitations of Chapter 2

There is a number of limitations associated with the work performed on this chapter. A single batch of iPSC-CMs was used to ascertain the optimum concentration of retinoic acid needed to generate atrial cardiomyocytes and the cells were only matured for 20 days. Batch to batch variability is a major limitation of iPSC-CMs and multiple differentiations are conventionally performed to mitigate this. iPSC-CMs are still maturing at day 20 and therefore are unlikely to have completed chamber specification (Lewandowski et al., 2018). Strict quality control thresholds were not put in place and may have exacerbated batch-to-batch variability.

6.1.1.2 Chapter 2 Future Work

The work performed in this chapter was sufficient to validate that the cells differentiated resembled atrial cardiomyocytes. There is a variety of other techniques that could have been performed to conclusively assess how ventricular or atrial like the iPSC-CMs derived were. Flow cytometry would have given more accurate percentages of MLC-2v/2a marker expression in the cells. RNA-seq and quantitative proteomics would have allowed comparative expression of a number of known compartment specific genes and proteins. Investigating expression of compartment specific markers in EHTs would have provided greater validation of the model.

6.1.2 Chapter 3: Generation of a human iPSC-derived cardiomyocyte/fibroblast engineered heart tissue model

A 3D co-culture cardiac model was required to assess the effect a clinically relevant pathogenic variant (TTNtv) had on structural remodelling. Structural remodelling refers to pathological changes in the morphology and structure of a chamber of the heart (Nattel et al., 2018). It is a defining characteristic of patients with DCM and thought to play a role in the pathology of TTNtv associated AF (Ahlberg et al., 2018). 2D iPSC-CM models have proven useful investigating the pathophysiology of principally monogenic diseases such as channelopathies (Garg et al., 2018). Their utility in modelling more complex heart diseases including cardiomyopathies and heart failure has been somewhat lacklustre. This is partially explained by the juxtaposition between the maturity of iPSC-CMs and the age dependent pathogenesis of complex cardiac disease. Primarily, however, it is due to 2D iPSC-CM models failing to recapitulate dysfunction in heterocellular cell to cell and cell to matrix interactions present in the 3D myocardium during disease. 3D co-culture cardiac models help remedy these limitations, as they confer increased contractile and electrophysiological maturity and a more physiologically relevant microenvironment (Mohr et al., 2022).

An engineered heart tissue (EHT) refers to a strip of cardiomyocytes embedded in matrix proteins. The strip is anchored between flexible silicone posts that provide preload. It is a commonly used 3D cardiac model and has been successfully employed to evaluate the phenotype of a variety of cardiomyopathies including dilated, mitochondrial, and hypertrophic (Breckwoldt et al., 2017) (Wang et al., 2014) (Hinson et al., 2015) (Cashman et al., 2016). Success of the model has been underpinned by its relative ease in generation and its practicality in providing robust measurements on contractile kinetics (Mannhardt et al., 2016). Chapter 3 describes the in-depth generation and incorporation of iPSC-CMs and iPSC-CFs into an EHT model. Some of the advantages of co-culture EHTs are shown in *Figure 6.2*. The derivation of quiescent but activatable iPSC-CFs is also demonstrated. Addition of iPSC-CFs into EHTs improved viability and caused an increase in tissue compaction. The increased tissue compaction was concomitant with an enhancement in contractile force. The results were consistent with previous studies by Rivera-Arbeláez et al., (2022) and Bargehr et al., (2019) which showed increased viability, tissue compaction and contractile force following the addition of ventricular cardiac fibroblasts or epicardial cells into stem cell derived cardiomyocyte EHT models. Improvements in tissue compaction and force were posited as being due to cardiac fibroblast mediated remodelling of the ECM in the tissue. A recent study, which investigated the effect addition of pro-epicardial cells had on EHTs, showed that it was the deposition of fibronectin from the stromal cells that caused the improved maturation and contractile function of the iPSC-CMs (Ong et al., 2023). Regardless of the mechanism, improvements in the contractile kinetics of 3D cardiac models are beneficial to recapitulating heart function *in vitro*.

The addition of iPSC-CFs to EHTs broadens the utility of the model. It does this by allowing researchers to study the interplay between different cell types in the heart. This is important as often these heterocellular interactions are disrupted during complex cardiac disease. There

are two advantages associated with using iPSC-CFs over primary cardiac fibroblasts in 3D cardiac models. The first is that iPSC-CFs are more accessible than healthy primary cardiac fibroblasts and more easily maintained in a quiescent state. As seen in *Chapter 5*, iPSC-CFs demonstrate greater plasticity for cardiac activation than their primary counterparts which likely helps facilitate the investigation of pro-fibrotic mechanisms. The second is that they permit the generation of cardiac models with a consistent genome. Genetic variants that cause pathogenic effect in different cell types, such as those found in patients with Fabry disease, could be explored using these isogenic models (Anastasakis et al., 2013). The addition of other non-myocyte cell types into EHTs would likely improve the ability of the model to recapitulate aspects of the human heart.

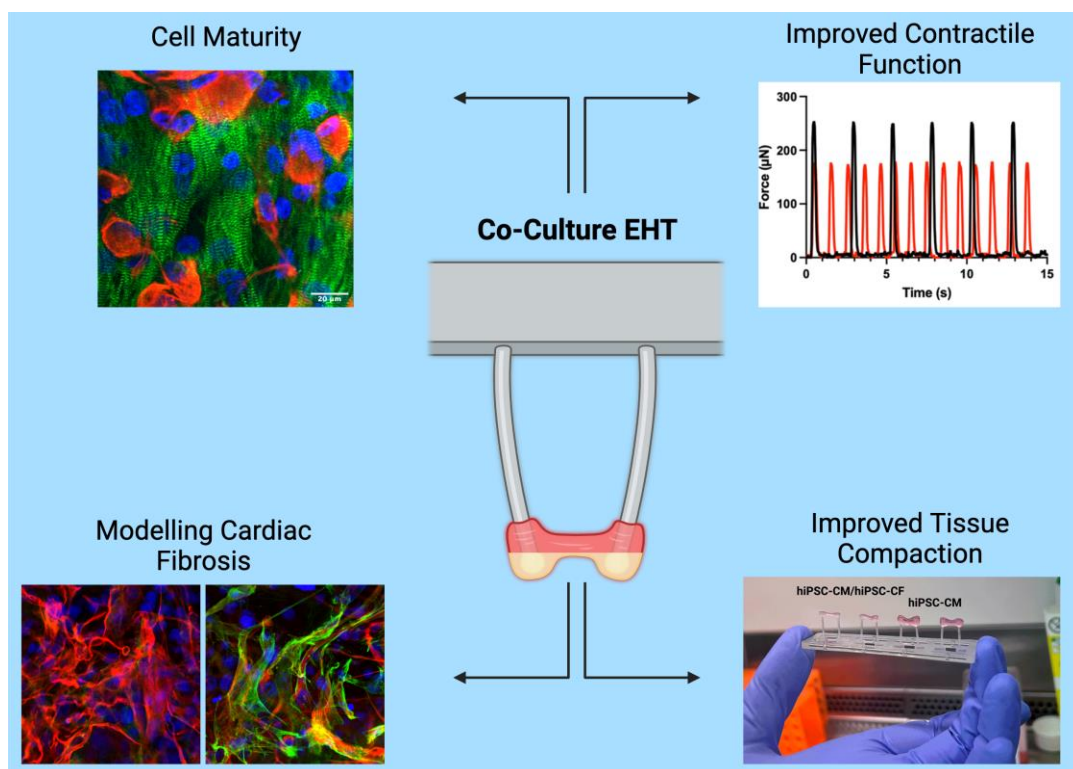


Figure 6.2: Benefits of Co-Culture EHTs

Co-culture EHTs containing iPSC-CMs and iPSC-CFs demonstrate increased tissue compaction and contractile function. The cardiomyocytes show anisotropic alignment and are capable of modelling aspects of cardiac fibrosis.

6.1.2.1 Limitations of Chapter 3

The work outlined in this chapter is consistent with previous studies that have proven that the addition of stromal cells to 3D cardiac models improves contractile function (Rivera-Arbeláez et al., 2022) (Bargehr et al., 2019). Assessing the activation status of the iPSC-CFs in the EHTs when cultured with and without the TGF- β 1 inhibitor would have improved the novelty of the study. Analysing contractile function in a greater number of EHT batches would have strengthened the data set. Strict thresholds on percentages of cells expressing a cardiac marker were not used.

6.1.2.2 Chapter 3 Future Work

There is a myriad of further experiments that could have been performed. Validating that TGF- β 1 inhibitor treatment of the EHTs prevented iPSC-CF activation would have improved the utility of the model in studying fibrotic diseases. Investigating the effects activation of iPSC-CFs had on signal propagation, using voltage mapping, would have confirmed whether the model could be used to investigate conduction heterogeneities caused by cardiac fibrosis. The addition of other non-myocyte cell types such as macrophages would have improved the physiological relevance of the model. However, such an approach should be performed iteratively, with extensive characterisation performed prior to the addition of a new cell type.

6.1.3 Chapter 4: A titin truncating variant causes changes in profibrotic signalling and response to cholinergic stimulation in cardiomyocytes

A founder variant in the gene *TTN*, described in Hoorntje et al., (2018), strongly predisposes individuals to early-onset AF and DCM. The pathogenic variant, which causes a putative TTNtv, was modelled in iPSCs using CRISPR-Cas9 gene editing. At the time of writing, no previously published studies have investigated the effect TTNtv have on the physiology of

atrial iPSC-CMs. Heterozygous *TTN*tv atrial iPSC-CMs demonstrated downregulation in *TTN* transcript expression, shortened sarcomeres and increased expression of cardiac stress markers. The cells showed increased beating frequencies and a greater susceptibility to cholinergic stimulation due to an upregulation in I_{KACH} . *In silico* simulation showed that despite being innocuous during sinus rhythm, dysregulation in cholinergic stimulation can act as a substrate for arrhythmia in tachycardia. *TTN*tv EHTs consisting of iPSC-CMs and iPSC-CFs showed increased expression and secretion of pro-fibrotic and extracellular matrix proteins including collagen 1 and fibronectin. Atrial fibrosis can act as a substrate for re-entry and can propitiate AF (Burnstein and Nattel, 2008).

6.1.3.1 *TTN*tv and DCM

The principal aim of this project was to explore the effect a *TTN*tv had on AF. Despite this, observations seen in a *TTN*tv atrial iPSC-CM model may yet provide insights into the pathology of DCM. Investigating the pathomechanism behind *TTN*tv DCM is difficult due to the practical challenges associated with studying a gene/protein the size of titin. Furthermore, the varied role the protein has in the sarcomere and the number of pathogenic variants identified in the transcript compound the problem and make identifying a unifying pathomechanism difficult. It is likely that the varied and often contrasting findings reported in previously published papers is due to these unique characteristics (Schafer et al., (2017) (McAfee et al., 2021) (Fomin et al., 2021) (Verdonschot et al., 2018) (Adams et al., 2019). Consistent with previous studies investigating *TTN*tv in rat models, the atrial iPSC-CMs demonstrated reduced gene expression of *TTN* (Schafer et al., 2017). Downregulated *TTN* transcript expression has been implicated as being causative in the development of *TTN*tv associated DCM through disruption to the cell cycle (Radke et al., 2019) (Liao et al., 2019). However, this was not explored during the course of this project. Recent studies that support

haploinsufficiency as a partial disease mechanism demonstrate no significant difference in gene expression or nonsense mediated decay of the truncated transcript (Fomin et al., 2021) (McAfee et al., 2021). Instead, insufficient availability of titin in the sarcomere was determined to be due to inefficient translation of the truncated transcript, or increased protein degradation. These studies however are performed on end stage TTNtv DCM hearts. Differences in *TTN* transcript expression may be present during the initial development of the disease.

Structural remodelling is a defining characteristic of DCM. No previous study has investigated the propensity for TTNtv to cause cardiac fibrosis in an iPSC-CM model. Upregulation of *PDGFA* and *PDGFB* in atrial iPSC-CMs likely caused the paracrine activation of nearby cardiac fibroblasts in the co-culture EHT model. Robust expression of *PDGFA* and *PDGFB* is seen in the ventricles, opening up the possibility that disruption of this signalling pathway is important in TTNtv associated ventricular remodelling and DCM pathogenesis (Gallini et al., 2016). No contractile dysfunction was observed in the TTNtv atrial EHTs. This is in contrast to previous studies investigating TTNtv in ventricular 3D iPSC-CM models, which observed diminished contractile force (Hinson et al., 2015) (Huang et al., 2023). Differences in the compartment specificity of the cardiomyocytes used, as well as the pathogenic variant modelled may explain these contrasting results.

6.1.3.2 *TTNtv associated Atrial Remodelling*

Pathogenic remodelling of the atria has now been observed in mouse and zebrafish TTNtv models (Kalstoe et al., 2023) (Ahlberg et al., 2018) (Huttner et al., 2018). Furthermore, recent studies show that DCM patients with TTNtv show greater left atrial dysfunction than non-carriers (Henkens et al., 2023). Atrial remodelling causes an increased risk for AF by acting as a substrate for re-entry. AF can cause mechanical stress in the atria and promote the

development of atrial fibrosis (Pellman et al., 2010). It is therefore unclear if the atrial remodelling seen in patients with TTNtv occurs before or after presentation of AF. Furthermore, it may be a ramification of left ventricular dysfunction. Previous studies have suggested the structural remodelling observed in patients with TTNtv as being tachycardia induced (Ahlberg et al., 2018). The increased AP frequencies observed in this study from the heterozygous TTNtv atrial iPSC-CMs is congruent with tachyarrhythmia induced fibrosis. The original hypothesis of the project was that the TTNtv would cause atrial fibrosis and consequently increase the risk of AF. This hypothesis suggested that TTNtv would cause shortened sarcomeres that confer mechanical dysfunction and stretch/stress. This would accompany an upregulation in cardiac stress markers and the activation of nearby cardiac fibroblasts in the heart. Evidence for some of the mechanisms delineated in this hypothesis is outlined in *Chapter 4*. An updated hypothesis based on the data derived in this project is illustrated in *Figure 6.3*. Significantly shortened sarcomeres were present in atrial TTNtv iPSC-CMs and this preceded an upregulation in the expression of the pro-fibrotic cardiac stress markers *PDGFA* and *PDGFB*. Co-culture EHT models showed increased secretion of extracellular matrix proteins, indicating greater propensity for atrial fibrosis. However contrary to what was expected, the TTNtv did not impede mechanical function, with the WT and heterozygous TTNtv EHTs demonstrating similar contractile kinetics. A recent study by Kellermayer et al., (2023) demonstrated that truncated titin isoforms are incorporated into the sarcomeres of primary human cardiomyocytes and cause minor structural and functional defects that can manifest during mechanical stretch. These defects were observed using super resolution microscopy. Compensation for these defects was thought to be the main driver for the development of DCM. It is possible that the incorporation of truncated titin into the sarcomeres of the atrial cardiomyocytes caused minor changes in contractile function which were imperceptible in an EHT model but still caused increased mechanical stress. iPSC-CMs

are conventionally matured for 30 days after the initiation of differentiation and show an immature phenotype similar to that of a neonatal cardiomyocyte (Yang et al., 2014). The average age at which DCM was diagnosed in patients with this TTNtv was 49 (Hoorntje et al., 2018). Contrasting timeframes/maturation of the cardiomyocytes may partly explain why mechanical dysfunction was not observed *in vitro*.

Signs of atrial remodelling were present in 40 % of the patients with the TTNtv described in Hoorntje et al., (2018). The patients who did not show signs of atrial remodelling (atrial dilatation) were still at risk of developing AF. This implies that structural remodelling of the atria may not be the defining mechanism causing AF in these patients and that a separate, electrical pathomechanism may be present.

6.1.3.3 TTNtv associated AF

The TTNtv atrial iPSC-CMs demonstrated broadly normal intracellular electrophysiology, with no significant changes in APD, upstroke velocity, maximum diastolic potential, inward sodium or outward potassium current. The cells showed an increased AP frequency, which is congruent with the pervasiveness of atrial and ventricular tachycardia in TTNtv patients (Hoorntje et al., 2018) (Verdonschot et al., 2018). The mechanism behind the greater AP frequency is uncertain. It may be due to the upregulation of the rate regulating potassium channel TWIK-1 (*KCNK1*). TWIK-1 is important in regulating heart rate and atrial morphology. Loss of function variants in this gene have caused bradyarrhythmia and atrial dilatation (Christensen et al., 2016). Overexpression may therefore lead to tachycardia. Increased expression of *KCNJ3*, which encodes a subunit of the acetylcholine-activated inward rectifier potassium current ($I_{K_{ACh}}$), was observed in the heterozygous atrial iPSC-CMs. Further examination demonstrated increased susceptibility to treatment with the acetylcholine agonist carbachol, with the heterozygous iPSC-CMs exhibiting significantly

greater APD shortening following treatment. *In silico* modelling of these changes proved to be pro-arrhythmic during sinus tachycardia. Greater expression of the I_{KACH} encoding gene *KCNJ3* has previously been shown in ventricular TTNtv iPSC-CMs (Huang et al., 2022). The two separate mechanisms proposed for TTNtv associated AF are illustrated in *Figure 6.3* and include: structural remodelling of the atria and dysregulated cholinergic stimulation. Atrial tachycardia, which was observed from analysis of the atrial iPSC-CMs spontaneous action potentials, can promote both mechanisms (Corradi et al., 2008) (Nattel et al., 2008).

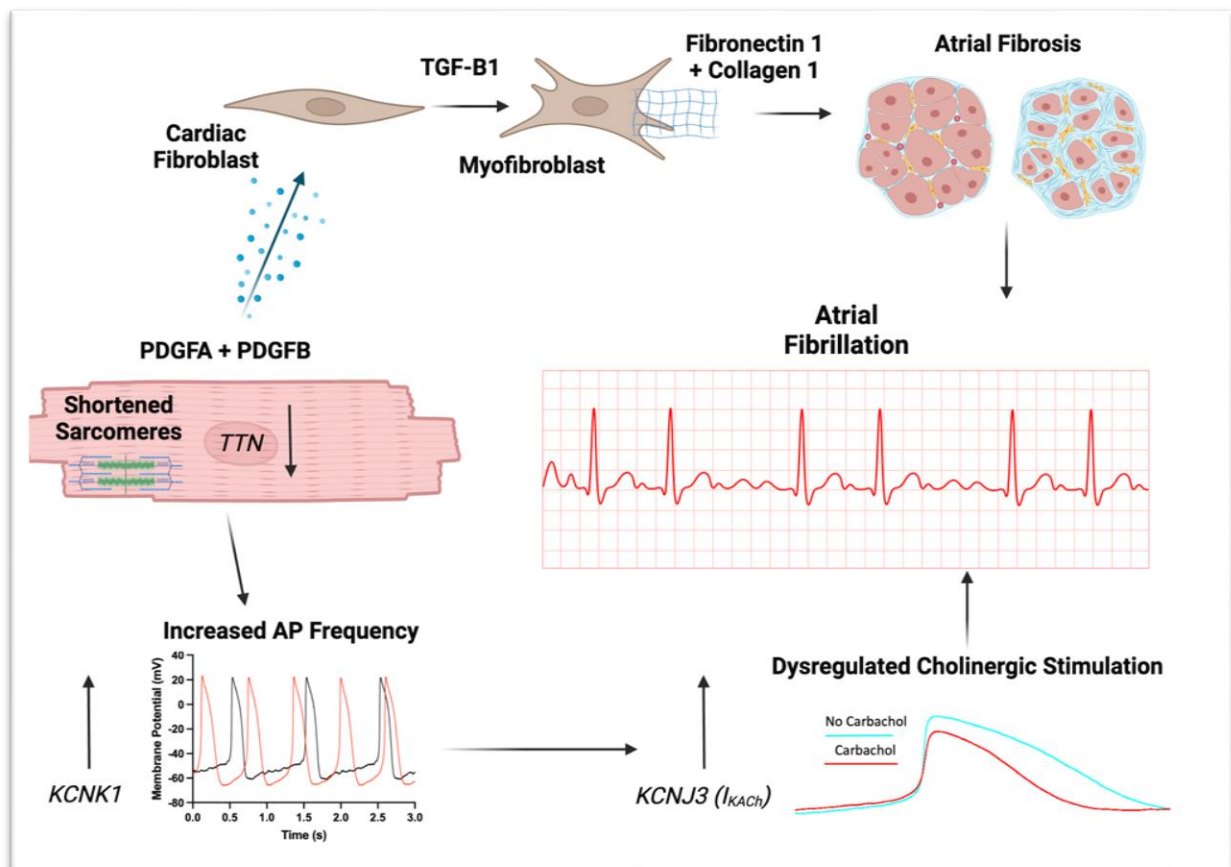


Figure 6.3: Mechanisms behind TTNtv associated Atrial Fibrillation

The TTNtv caused a reduction in *TTN* gene expression and the formation of shortened sarcomeres. An increase in *PDGFA* and *PDGFB* expression caused TGF-β1 mediated activation of nearby cardiac fibroblasts in an EHT model. Activated cardiac fibroblasts secreted more fibronectin and collagen, indicating an increased propensity for fibrosis. Atrial fibrosis is a substrate for arrhythmia. An increase in *KCNK1* expression may have caused the increased AP frequencies observed in TTNtv atrial iPSC-CMs. Upregulated expression of *KCNJ3* caused increased sensitivity to the acetylcholine agonist carbachol. This dysregulation in cholinergic stimulation proved arrhythmogenic when modelled *in silico*.

6.1.3.4 Limitations of Chapter 4

There are a variety of ways in which the data presented in this chapter could have been improved. 2D and 3D iPSC-CMs are structurally, transcriptionally, and electrophysiologically immature and may therefore not be an ideal model in which to investigate age dependent diseases such as DCM or AF. The translation and incorporation of the truncated titin protein into the sarcomeres of cardiomyocytes was not explored. Therefore, I cannot rule out the possibility that the changes listed above were not due to differences in differentiation efficiency (maturity/types of cells derived) caused by reduced *TTN* gene expression.

The exact mechanisms by which the *TTN*tv caused the upregulation of *PDGFA* and *PDGFB* was not determined. Furthermore, secreted protein levels of PDGFA and PDGFB should have been measured in the iPSC-CM supernatant to confirm the increase in expression. The increased expression of these pro-fibrotic genes was hypothesised to be due to mechanical stress caused by shortened sarcomeres. However, disruption to mechanical function was not observed in the EHTs. Furthermore, it is currently unclear as to why the *TTN*tv would cause increased activity of I_{KACH} . Changes in intracellular calcium handling are commonly observed in AF but were not explored in the atrial iPSC-CMs. Disruption to intracellular calcium handling represents a viable but unexamined mechanism for *TTN*tv associated AF and may explain the increased AP frequencies.

6.1.3.5 Chapter 4 Future Work

Mechanical dysfunction is a hallmark of pathogenic remodelling but was not detected in the *TTN*tv EHTs. Investigating contractile function using a more precise technique designed to assess sarcomere mechanics (e.g. SarcTrack) may help detect slight changes in contractility caused by *TTN*tv in atrial iPSC-CMs (Toepfer et al., 2019). Measurement of I_{KACH} was not

performed during this project but would help confirm parasympathetic dysregulation as the mechanism of arrhythmia. Approximately half of all the patients with the pathogenic TTNtv in Hoorntje et al., (2018) had at least one risk factor (e.g. hypertension, coronary artery disease, diabetes, dyslipidaemia). Exposing the atrial TTNtv iPSC-CMs to a so called ‘second hit’, such as the tachypacing outlined in Seibert et al., (2023) may help manifest a more blatant arrhythmogenic phenotype.

6.1.4 Chapter 5: Investigating the physiological relevance and utility of induced pluripotent stem cell derived cardiac fibroblasts

iPSC-CFs represent a new and exciting model to evaluate mechanisms behind cardiac fibrosis *in vitro*. As mentioned in 6.1.2, iPSC-CFs are a more practical model than their primary counterparts, as they are easier to maintain in a quiescent state and show greater plasticity for activation than primary fibroblasts. Previous studies have indicated that they show similarities to primary ventricular cardiac fibroblasts in their transcriptional, structural and functional characteristics (Moita et al., 2022). However further experimental evidence on the physiological relevance of iPSC-CFs is required to convince many of their utility in cardiac disease modelling.

Relatively little is known about the electrophysiological characteristics of cardiac fibroblasts and myofibroblasts, making it an interesting and potentially substantive field of study.

Changes in ionic current are thought to be important in the activation of cardiac fibroblasts and the development of fibrosis (Zhang et al., 2021). Depolarisation of myofibroblasts occurs due to changes in ion current during activation. This can be pro-arrhythmic in electrotonically coupled cardiomyocytes, as a more positive membrane potential can impede conduction and increase excitability (Ongstad and Kohl, 2016). There is still however a lack of agreement on the currents that are functionally expressed in cardiac fibroblasts and how they change during

cardiac fibrosis (Xing et al., 2023). Chapter 5 attempted to validate the utility of iPSC-CFs as a cardiac disease model by exploring changes in electrophysiology during fibroblast activation.

The iPSC-CFs and ventricular cardiac fibroblasts (VCFs) showed activation following treatment with TGF- β 1 and similar responses in the expression of voltage-gated ion channel subunit genes. Voltage-gated ion channel subunit genes which showed interesting and consistent responses to TGF- β 1 treatment in iPSC-CFs and VCFs included *CACNA1C*, *KCNB1*, *KCNJ2* and *KCNMA1*. Expression of mechanosensitive ion channel genes, such as those that encode TRP and piezo channels, was dysregulated in both cell types. This was probably due to prolonged culture of the cells on stiff plastic surfaces (Gilles et al., 2020). RNA-seq and quantitative proteomics analysis of iPSC-CFs treated with TGF- β 1 demonstrated pro-fibrotic changes in gene and protein expression that were likely orchestrated by upregulated TGFBI and CTGF. The cells showed increased expression of the ion channel subunit genes *KCNG1* and *KCTD16* following TGF- β 1 treatment. *KCNG1* encodes an ion channel subunit (K_v6.1) that interacts and modulates activity of the membrane potential regulating channel K_v2.1 (*KCNB1*), whose expression was previously shown to be downregulated in qPCR experiments (Kramer et al., 1998). Further functional experiments exploring the role of *KCNG1* (K_v6.1) on cardiac fibroblast activation may help elucidate this interaction.

The atrial cardiac fibroblasts (ACFs) used in this study demonstrated minimal activation following TGF- β 1 treatment. Their inclusion as a primary control to the iPSC-CFs in RNA-seq and quantitative proteomics experiments was in hindsight suboptimal. The ACFs, which were purchased from a supplier, showed atypical responses to cardiac fibroblast activation. Difficulty obtaining healthy quiescent cardiac fibroblasts from industrial biotechnology companies highlights the potential utility of iPSC-CFs in cardiac disease modelling.

6.1.4.1 Limitations of Chapter 5

The VCFs used in this chapter were from an explanted heart, making them an unsuitable healthy primary cardiac fibroblast control. The validity of the data derived using the VCFs is therefore dubious. Cardiac fibroblasts are mechanosensitive cells that respond to slight changes in stiffness in the human heart. Prolonged culture of the cells on unphysiologically stiff plastic surfaces likely causes disruption to their mechanosensitivity and activation mechanisms (Gilles et al., 2022). This is evidenced by the erratic and unexpected changes in mechanosensitive ion channel expression observed in iPSC-CFs and VCFs following TGF- β 1 treatment.

The ACFs used in this study behaved atypically to TGF- β 1 treatment. The ACFs did not demonstrate sufficient α -SMA expression following TGF- β 1 treatment and therefore should not have been used as a control to the iPSC-CFs in the RNA-seq and quantitative proteomics experiments. In retrospect, quantitative proteomics may not have been the ideal technique to identify changes in ion channel subunit expression, as it lacked the sensitivity to identify lowly expressed ion channel subunits (Pappireddi et al., 2019).

6.1.4.2 Chapter 5 Future Work

Future work investigating the utility of iPSC-CFs should endeavour to use freshly derived cells where possible to mitigate some of the limitations listed above. Despite this, several interesting changes in ion channel subunit gene expression were identified in the iPSC-CFs following TGF- β 1 treatment. Activity of the K_v2.1, K_{ir}2.1 and CaV1.2 channels should be measured in cardiac fibroblasts prior to and following TGF- β 1 treatment, to validate the changes observed in the gene expression of *KCNB1*, *KCNG1*, *KCNJ2* and *CACNA1C*. Furthermore, the membrane potential of cardiac fibroblasts should also be measured prior and

after cardiac fibroblast activation to assess whether changes in these channels contribute towards myofibroblast depolarisation. Future experiments should utilise soft substrates to study mechanosensitive ion channel gene expression and function (Hall et al., 2023).

6.2 Concluding Remarks

Cardiovascular researchers are still intrigued by the potential iPSC-CMs have in cardiac disease modelling, 14 years after their conception (Zwi et al., 2009). Stem cell based cardiac disease modelling, which is still considered to be in its infancy, has made substantial progress in the past decade. The development of cheaper and more efficient differentiation protocols has coincided with newfound techniques that can be used to derive compartment specific cell types such as atrial cardiomyocytes. This has broadened the appeal and utility of the model to otherwise sceptical users.

The immaturity of the cells generated is still a major hinderance but is being iteratively improved by a variety of maturation strategies including soft substrates, electrical pacing, fatty acid enriched media compositions and 3D cardiac models (Thomas et al., 2022). The development and use of iPSC derived co-culture 3D cardiac models has been of particular benefit in exploring the pathophysiology of more complex cardiac diseases and is quickly becoming standard practise in the field. Moreover, iPSC-CFs have emerged as an exciting model that can be used independently or as part of an isogenic system to explore cardiac fibrosis. Researchers are now aware of the limitations of iPSC-CM models and are mitigating some of these by augmenting work performed *in vitro* with computational modelling. This combined *in vitro/in silico* approach will likely continue to promote the replacement of *in vivo* models in cardiac disease modelling.

The link between TTNtv and AF is now well established. This thesis identified structural remodelling and dysregulation in cholinergic stimulation as two potential mechanisms by

which TTNtv can cause AF in an atrial iPSC-CM model. Tachycardia seems integral to the pathogenesis of TTNtv associated AF and likely concatenates these two mechanisms. Enhanced understanding of the mechanisms behind TTNtv associated AF will ultimately improve patient care through the development of novel target specific therapeutics.

Chapter 7 Bibliography

1. Abubakar, I.I., Tillmann, T. and Banerjee, A., 2015. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*, 385(9963), pp.117-171.
2. Acevedo-Acevedo, S. and Crone, W.C., 2015. Substrate stiffness effect and chromosome missegregation in hIPS cells. *Journal of Negative Results in Biomedicine*, 14, pp.1-7.
3. Adams, M., Fleming, J.R., Riehle, E., Zhou, T., Zacharchenko, T., Markovic, M. and Mayans, O., 2019. Scalable, Non-denaturing Purification of Phosphoproteins Using Ga³⁺-IMAC: N2A and M1M2 Titin Components as Study case. *The protein journal*, 38(2), pp.181-189.
4. Agarkova, I. and Perriard, J.C., 2005. The M-band: an elastic web that crosslinks thick filaments in the center of the sarcomere. *Trends in cell biology*, 15(9), pp.477-485.
5. Ahlberg, G., Refsgaard, L., Lundegaard, P.R., Andreasen, L., Ranthe, M.F., Linscheid, N., Nielsen, J.B., Melbye, M., Haunsø, S., Sajadieh, A. and Camp, L., 2018. Rare truncating variants in the sarcomeric protein titin associate with familial and early-onset atrial fibrillation. *Nature communications*, 9(1), pp.1-11.
6. Ahmad, F.S., Jin, Y., Grassam-Rowe, A., Zhou, Y., Yuan, M., Fan, X., Zhou, R., Mu-U-Min, R., O'Shea, C., Ibrahim, A.M. and Hyder, W., 2023. Generation of cardiomyocytes from human-induced pluripotent stem cells resembling atrial cells with ability to respond to adrenoceptor agonists. *Philosophical Transactions of the Royal Society B*, 378(1879), p.20220312.
7. Ahn, M.S., Eom, Y.W., Oh, J.E., Cha, S.K., Park, K.S., Son, J.W., Lee, J.W., Youn, Y.J., Ahn, S.G., Kim, J.Y. and Lee, S.H., 2020. Transient receptor potential channel TRPV4 mediates TGF- β 1-induced differentiation of human ventricular fibroblasts. *Cardiology Journal*, 27(2), pp.162-170.
8. Akinrinade, O., Koskenvuo, J.W. and Alastalo, T.P., 2015. Prevalence of titin truncating variants in general population. *PloS one*, 10(12), p.e0145284.
9. Akinrinade, O., Ollila, L., Vattulainen, S., Tallila, J., Gentile, M., Salmenperä, P., Koillinen, H., Kaartinen, M., Nieminen, M.S., Myllykangas, S. and Alastalo, T.P., 2015. Genetics and genotype–phenotype correlations in Finnish patients with dilated cardiomyopathy. *European heart journal*, 36(34), pp.2327-2337.

10. Al-Khatib, S.M., Allen LaPointe, N.M., Chatterjee, R., Crowley, M.J., Dupre, M.E., Kong, D.F., Lopes, R.D., Povsic, T.J., Raju, S.S., Shah, B. and Kosinski, A.S., 2014. Rate-and rhythm-control therapies in patients with atrial fibrillation: a systematic review. *Annals of internal medicine*, 160(11), pp.760-773.
11. Ali, D.C., Naveed, M., Gordon, A., Majeed, F., Saeed, M., Ogbuke, M.I., Atif, M., Zubair, H.M. and Changxing, L., 2020. β -Adrenergic receptor, an essential target in cardiovascular diseases. *Heart failure reviews*, 25, pp.343-354.
12. Alloui, A., Zimmermann, K., Mamet, J., Duprat, F., Noel, J., Chemin, J., Guy, N., Blondeau, N., Voilley, N., Rubat-Coudert, C. and Borsotto, M., 2006. TREK-1, a K⁺ channel involved in polymodal pain perception. *The EMBO journal*, 25(11), pp.2368-2376.
13. Amano, Y., Nishiguchi, A., Matsusaki, M., Iseoka, H., Miyagawa, S., Sawa, Y., Seo, M., Yamaguchi, T. and Akashi, M., 2016. Development of vascularized iPSC derived 3D-cardiomyocyte tissues by filtration Layer-by-Layer technique and their application for pharmaceutical assays. *Acta biomaterialia*, 33, pp.110-121.
14. Amodeo, P., Fraternali, F., Lesk, A.M. and Pastore, A., 2001. Modularity and homology: modelling of the titin type I modules and their interfaces. *Journal of molecular biology*, 311(2), pp.283-296.
15. Amos, G.J., Wettwer, E., Metzger, F., Li, Q., Himmel, H.M. and Ravens, U., 1996. Differences between outward currents of human atrial and subepicardial ventricular myocytes. *The Journal of physiology*, 491(1), pp.31-50.
16. An, Z., Yang, G., Liu, X., Zhang, Z. and Liu, G., 2018. New progress in understanding the cellular mechanisms of anti-arrhythmic drugs. *Open life sciences*, 13(1), pp.335-339.
17. Anastasakis, A., Papatheodorou, E. and Klavdios Steriotis, A., 2013. Fabry disease and cardiovascular involvement. *Current pharmaceutical design*, 19(33), pp.5997-6008.
18. Andersen, J.H. and Olesen, M.S., 2019. Genetics of atrial fibrillation and atrial cardiomyopathy. *Journal of Human and Clinical Genetics*, 1(2).
19. Andersen, J.H., Andreassen, L. and Olesen, M.S., 2021. Atrial fibrillation—a complex polygenetic disease. *European Journal of Human Genetics*, 29(7), pp.1051-1060.
20. Anderson, R.H., Yanni, J., Boyett, M.R., Chandler, N.J. and Dobrzynski, H., 2009. The anatomy of the cardiac conduction system. *Clinical Anatomy: The Official Journal of the American Association of Clinical Anatomists and the British Association of Clinical Anatomists*, 22(1), pp.99-113.

21. András, V., Tomek, J., Nagy, N., Virág, L., Passini, E., Rodriguez, B. and Baczkó, I., 2021. Cardiac transmembrane ion channels and action potentials: cellular physiology and arrhythmogenic behavior. *Physiological reviews*.
22. Andreasen, L., Bertelsen, L., Ghouse, J., Lundegaard, P.R., Ahlberg, G., Refsgaard, L., Rasmussen, T.B., Eiskjær, H., Haunsø, S., Vejlstup, N. and Svendsen, J.H., 2020. Early-onset atrial fibrillation patients show reduced left ventricular ejection fraction and increased atrial fibrosis. *Scientific reports*, 10(1), pp.1-8.
23. Ang, Y.S., Rajamani, S., Haldar, S.M. and Hueser, J., 2020. A new therapeutic framework for atrial fibrillation drug development. *Circulation research*, 127(1), pp.184-201.
24. Ang, Y.S., Rajamani, S., Haldar, S.M. and Hueser, J., 2020. A new therapeutic framework for atrial fibrillation drug development. *Circulation research*, 127(1), pp.184-201.
25. Anter, E., Jessup, M. and Callans, D.J., 2009. Atrial fibrillation and heart failure: treatment considerations for a dual epidemic. *Circulation*, 119(18), pp.2516-2525.
26. Arboix, A. and Alioc, J., 2010. Cardioembolic stroke: clinical features, specific cardiac disorders and prognosis. *Current cardiology reviews*, 6(3), pp.150-161.
27. Argenziano, M., Lambers, E., Hong, L., Sridhar, A., Zhang, M., Chalazan, B., Menon, A., Savio-Galimberti, E., Wu, J.C., Rehman, J. and Darbar, D., 2018. Electrophysiologic characterization of calcium handling in human induced pluripotent stem cell-derived atrial cardiomyocytes. *Stem cell reports*, 10(6), pp.1867-1878.
28. Aujla, P.K. and Kassiri, Z., 2021. Diverse origins and activation of fibroblasts in cardiac fibrosis. *Cellular signalling*, 78, p.109869.
29. Bae, H., Kim, T. and Lim, I., 2020. Effects of nitric oxide on apoptosis and voltage-gated calcium channels in human cardiac myofibroblasts. *Clinical and Experimental Pharmacology and Physiology*, 47(1), pp.16-26.
30. Banerjee, I., Fuseler, J.W., Price, R.L., Borg, T.K. and Baudino, T.A., 2007. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *American Journal of Physiology-Heart and Circulatory Physiology*, 293(3), pp.H1883-H1891.
31. Bang, M.L., Centner, T., Fornoff, F., Geach, A.J., Gotthardt, M., McNabb, M., Witt, C.C., Labeit, D., Gregorio, C.C., Granzier, H. and Labeit, S., 2001. The complete gene sequence of titin, expression of an unusual ≈ 700 -kDa titin isoform, and its interaction

- with obscurin identify a novel Z-line to I-band linking system. *Circulation research*, 89(11), pp.1065-1072.
32. Bar-Peled, L. and Sabatini, D.M., 2014. Regulation of mTORC1 by amino acids. *Trends in cell biology*, 24(7), pp.400-406.
 33. Bargehr, J., Ong, L.P., Colzani, M., Davaapil, H., Hofsteen, P., Bhandari, S., Gambardella, L., Le Novère, N., Iyer, D., Sampaziotis, F. and Weinberger, F., 2019. Epicardial cells derived from human embryonic stem cells augment cardiomyocyte-driven heart regeneration. *Nature biotechnology*, 37(8), pp.895-906.
 34. Barold, S.S., 2003. Willem Einthoven and the birth of clinical electrocardiography a hundred years ago. *Cardiac electrophysiology review*, 7(1), pp.99-104.
 35. Barrett, K.M.S., Cirulli, E.T., Bolze, A., Rowan, C., Elhanan, G., Grzymalski, J.J., Lee, W. and Washington, N.L., 2023. Cardiomyopathy prevalence exceeds 30% in individuals with TTN variants and early atrial fibrillation. *Genetics in Medicine*, 25(4), p.100012.
 36. Barton, A.K., McGowan, M., Smyth, A., Wright, G.A. and Gardner, R.S., 2020. Classification and choice of antiarrhythmic therapies. *Prescriber*, 31(3), pp.11-17.
 37. Bayer, J.D., Boukens, B.J., Krul, S.P., Roney, C.H., Driessen, A.H., Berger, W.R., Van den Berg, N.W., Verkerk, A.O., Vigmond, E.J., Coronel, R. and de Groot, J.R., 2019. Acetylcholine delays atrial activation to facilitate atrial fibrillation. *Frontiers in physiology*, 10, p.1105.
 38. Beauchamp, P., Jackson, C.B., Ozhatil, L.C., Agarkova, I., Galindo, C.L., Sawyer, D.B., Suter, T.M. and Zuppinger, C., 2020. 3D co-culture of iPSC-derived cardiomyocytes with cardiac fibroblasts improves tissue-like features of cardiac spheroids. *Frontiers in molecular biosciences*, 7, p.14.
 39. Beauchamp, P., Moritz, W., Kelm, J.M., Ullrich, N.D., Agarkova, I., Anson, B.D., Suter, T.M. and Zuppinger, C., 2015. Development and characterization of a scaffold-free 3D spheroid model of induced pluripotent stem cell-derived human cardiomyocytes. *Tissue Engineering Part C: Methods*, 21(8), pp.852-861.
 40. Beckmann, J.S. and Spencer, M., 2008. Calpain 3, the “gatekeeper” of proper sarcomere assembly, turnover and maintenance. *Neuromuscular Disorders*, 18(12), pp.913-921.
 41. Begay, R.L., Graw, S., Sinagra, G., Merlo, M., Slavov, D., Gowan, K., Jones, K.L., Barbati, G., Spezzacatene, A., Brun, F. and Di Lenarda, A., 2015. Role of titin missense variants in dilated cardiomyopathy. *Journal of the American Heart Association*, 4(11), p.e002645.

42. Benjamin, I.J., Jalil, J.E., Tan, L.B., Cho, K., Weber, K.T. and Clark, W.A., 1989. Isoproterenol-induced myocardial fibrosis in relation to myocyte necrosis. *Circulation research*, 65(3), pp.657-670.
43. Bhattacharya, S., Burridge, P.W., Kropp, E.M., Chuppa, S.L., Kwok, W.M., Wu, J.C., Boheler, K.R. and Gundry, R.L., 2014. High efficiency differentiation of human pluripotent stem cells to cardiomyocytes and characterization by flow cytometry. *JoVE (Journal of Visualized Experiments)*, (91), p.e52010.
44. Bielecka-Dabrowa, A., Mikhailidis, D.P., Rysz, J. and Banach, M., 2009. The mechanisms of atrial fibrillation in hyperthyroidism. *Thyroid research*, 2, pp.1-7.
45. Bizhanov, K.A., Abzaliyev, K.B., Baimbetov, A.K., Sarsenbayeva, A.B. and Lyan, E., 2023. Atrial fibrillation: Epidemiology, pathophysiology, and clinical complications (literature review). *Journal of Cardiovascular Electrophysiology*, 34(1), pp.153-165.
46. Bizy, A., Guerrero-Serna, G., Hu, B., Ponce-Balbuena, D., Willis, B.C., Zarzoso, M., Ramirez, R.J., Sener, M.F., Mundada, L.V., Klos, M. and Devaney, E.J., 2013. Myosin light chain 2-based selection of human iPSC-derived early ventricular cardiac myocytes. *Stem cell research*, 11(3), pp.1335-1347.
47. Blechschmidt, S., Haufe, V., Benndorf, K. and Zimmer, T., 2008. Voltage-gated Na⁺ channel transcript patterns in the mammalian heart are species-dependent. *Progress in biophysics and molecular biology*, 98(2-3), pp.309-318.
48. Bondue, A., Arbustini, E., Bianco, A., Ciccarelli, M., Dawson, D., De Rosa, M., Hamdani, N., Hilfiker-Kleiner, D., Meder, B., Leite-Moreira, A.F. and Thum, T., 2018. Complex roads from genotype to phenotype in dilated cardiomyopathy: scientific update from the Working Group of Myocardial Function of the European Society of Cardiology. *Cardiovascular research*, 114(10), pp.1287-1303.
49. Borisov, A.B., Kontogianni-Konstantopoulos, A., Bloch, R.J., Westfall, M.V. and Russell, M.W., 2004. Dynamics of obscurin localization during differentiation and remodeling of cardiac myocytes: obscurin as an integrator of myofibrillar structure. *Journal of Histochemistry & Cytochemistry*, 52(9), pp.1117-1127.
50. Breckwoldt, K., Letuffe-Brenière, D., Mannhardt, I., Schulze, T., Ulmer, B., Werner, T., Benzin, A., Klampe, B., Reinsch, M.C., Laufer, S. and Shibamiya, A., 2017. Differentiation of cardiomyocytes and generation of human engineered heart tissue. *Nature protocols*, 12(6), pp.1177-1197.
51. Brieler, J.A.Y., Breeden, M.A. and Tucker, J., 2017. Cardiomyopathy: an overview. *American family physician*, 96(10), pp.640-646.

52. Buffolo, F., Tetti, M., Mulatero, P. and Monticone, S., 2022. Aldosterone as a mediator of cardiovascular damage. *Hypertension*, 79(9), pp.1899-1911.
53. Buikema, J.W., Van Der Meer, P., Sluijter, J.P. and Domian, I.J., 2013. Concise review: engineering myocardial tissue: the convergence of stem cells biology and tissue engineering technology. *Stem Cells*, 31(12), pp.2587-2598.
54. Burke, R.M., Lighthouse, J.K., Mickelsen, D.M. and Small, E.M., 2019. Sacubitril/valsartan decreases cardiac fibrosis in left ventricle pressure overload by restoring PKG signaling in cardiac fibroblasts. *Circulation: Heart Failure*, 12(4), p.e005565.
55. BurrIDGE, P.W., Matsa, E., Shukla, P., Lin, Z.C., Churko, J.M., Ebert, A.D., Lan, F., Diecke, S., Huber, B., Mordwinkin, N.M. and Plews, J.R., 2014. Chemically defined generation of human cardiomyocytes. *Nature methods*, 11(8), pp.855-860.
56. Burstein, B. and Nattel, S., 2008. Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation. *Journal of the American College of Cardiology*, 51(8), pp.802-809.
57. Burstein, B., Libby, E., Calderone, A. and Nattel, S., 2008. Differential behaviors of atrial versus ventricular fibroblasts: a potential role for platelet-derived growth factor in atrial-ventricular remodeling differences. *Circulation*, 117(13), pp.1630-1641.
58. Camacho, P., Fan, H., Liu, Z. and He, J.Q., 2016. Large mammalian animal models of heart disease. *Journal of cardiovascular development and disease*, 3(4), p.30.
59. Carmignac, V., Salih, M.A., Quijano-Roy, S., Marchand, S., Al Rayess, M.M., Mukhtar, M.M., Urtizberea, J.A., Labeit, S., Guicheney, P., Leturcq, F. and Gautel, M., 2007. C-terminal titin deletions cause a novel early-onset myopathy with fatal cardiomyopathy. *Annals of neurology*, 61(4), pp.340-351.
60. Cashman, T.J., Josowitz, R., Johnson, B.V., Gelb, B.D. and Costa, K.D., 2016. Human engineered cardiac tissues created using induced pluripotent stem cells reveal functional characteristics of BRAF-mediated hypertrophic cardiomyopathy. *PloS one*, 11(1), p.e0146697.
61. Celotto, C., Sánchez, C., Abdollahpur, M., Sandberg, F., Rodriguez, J.F., Laguna, P. and Pueyo, E., 2022, September. Effects of Acetylcholine Release Spatial Distribution on the Frequency of Atrial Reentrant Circuits: a Computational Study. In *2022 Computing in Cardiology (CinC)* (Vol. 498, pp. 1-4). IEEE.
62. Cha, T.J., Ehrlich, J.R., Chartier, D., Qi, X.Y., Xiao, L. and Nattel, S., 2006. Kir3-based inward rectifier potassium current: potential role in atrial tachycardia remodeling effects on atrial repolarization and arrhythmias. *Circulation*, 113(14), pp.1730-1737.

63. Chang, M., Gada, K.D., Chidipi, B., Tsalatsanis, A., Gibbons, J., Remily-Wood, E., Logothetis, D.E., Oberstaller, J. and Noujaim, S.F., 2022. IKACH is constitutively active via PKC epsilon in aging mediated atrial fibrillation. *Iscience*, 25(11).
64. Chatelier, A., Mercier, A., Tremblier, B., Thériault, O., Moubarak, M., Benamer, N., Corbi, P., Bois, P., Chahine, M. and Faivre, J.F., 2012. A distinct de novo expression of Nav1.5 sodium channels in human atrial fibroblasts differentiated into myofibroblasts. *The Journal of Physiology*, 590(17), pp.4307-4319.
65. Chavali, N.V., Kryshnal, D.O., Parikh, S.S., Wang, L., Glazer, A.M., Blackwell, D.J., Kroncke, B.M., Shoemaker, M.B. and Knollmann, B.C., 2019. The patient-independent human iPSC model—a new tool for rapid determination of genetic variant pathogenicity in long QT syndrome. *Heart rhythm*, 16(11), p.1686.
66. Chen, J.B., Tao, R., Sun, H.Y., Tse, H.F., Lau, C.P. and Li, G.R., 2010. Multiple Ca²⁺ signaling pathways regulate intracellular Ca²⁺ activity in human cardiac fibroblasts. *Journal of cellular physiology*, 223(1), pp.68-75.
67. Chen, S.N., Lam, C.K., Wan, Y.W., Gao, S., Malak, O.A., Zhao, S.R., Lombardi, R., Ambardekar, A.V., Bristow, M.R., Cleveland, J. and Gigli, M., 2022. Activation of PDGFRA signaling contributes to filamin C-related arrhythmogenic cardiomyopathy. *Science Advances*, 8(8), p.eabk0052.
68. Chen, Z., Xian, W., Bellin, M., Dorn, T., Tian, Q., Goedel, A., Dreizehnter, L., Schneider, C.M., Ward-van Oostwaard, D., Ng, J.K.M. and Hinkel, R., 2017. Subtype-specific promoter-driven action potential imaging for precise disease modelling and drug testing in iPSC-derived cardiomyocytes. *European heart journal*, 38(4), pp.292-301.
69. Cho, K.W., Lee, W.H., Kim, B.S. and Kim, D.H., 2020. Sensors in heart-on-a-chip: A review on recent progress. *Talanta*, 219, p.121269.
70. Choi, S.H., Weng, L.C., Roselli, C., Lin, H., Haggerty, C.M., Shoemaker, M.B., Barnard, J., Arking, D.E., Chasman, D.I., Albert, C.M. and Chaffin, M., 2018. Association between titin loss-of-function variants and early-onset atrial fibrillation. *Jama*, 320(22), pp.2354-2364.
71. Chow, E., Bernjak, A., Williams, S., Fawdry, R.A., Hibbert, S., Freeman, J., Sheridan, P.J. and Heller, S.R., 2014. Risk of cardiac arrhythmias during hypoglycemia in patients with type 2 diabetes and cardiovascular risk. *Diabetes*, 63(5), pp.1738-1747.
72. Christensen, A.H., Chatelain, F.C., Huttner, I.G., Olesen, M.S., Soka, M., Feliciani, S., Horvat, C., Santiago, C.F., Vandenberg, J.I., Schmitt, N. and Olesen, S.P., 2016. The two-

- pore domain potassium channel, TWIK-1, has a role in the regulation of heart rate and atrial size. *Journal of molecular and cellular cardiology*, 97, pp.24-35.
73. Christophersen, I.E. and Ellinor, P.T., 2016. Genetics of atrial fibrillation: from families to genomes. *Journal of human genetics*, 61(1), pp.61-70.
 74. Christophersen, I.E., Olesen, M.S., Liang, B., Andersen, M.N., Larsen, A.P., Nielsen, J.B., Haunsø, S., Olesen, S.P., Tveit, A., Svendsen, J.H. and Schmitt, N., 2013. Genetic variation in KCNA5: impact on the atrial-specific potassium current I_{Kur} in patients with lone atrial fibrillation. *European heart journal*, 34(20), pp.1517-1525.
 75. Christophersen, I.E., Rienstra, M., Roselli, C., Yin, X., Geelhoed, B., Barnard, J., Lin, H., Arking, D.E., Smith, A.V., Albert, C.M. and Chaffin, M., 2017. Large-scale analyses of common and rare variants identify 12 new loci associated with atrial fibrillation. *Nature genetics*, 49(6), pp.946-952.
 76. Chugh, S.S., Havmoeller, R., Narayanan, K., Singh, D., Rienstra, M., Benjamin, E.J., Gillum, R.F., Kim, Y.H., McAnulty Jr, J.H., Zheng, Z.J. and Forouzanfar, M.H., 2014. Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. *Circulation*, 129(8), pp.837-847.
 77. Cojan-Minzat, B.O., Zlibut, A. and Agoston-Coldea, L., 2021. Non-ischemic dilated cardiomyopathy and cardiac fibrosis. *Heart Failure Reviews*, 26, pp.1081-1101.
 78. Colombo, F., Noël, J., Mayers, P., Mercier, I. and Calderone, A., 2001. β -Adrenergic stimulation of rat cardiac fibroblasts promotes protein synthesis via the activation of phosphatidylinositol 3-kinase. *Journal of molecular and cellular cardiology*, 33(6), pp.1091-1106.
 79. Comtois, P., Kneller, J. and Nattel, S., 2005. Of circles and spirals: bridging the gap between the leading circle and spiral wave concepts of cardiac reentry. *EP Europace*, 7(s2), pp.S10-S20.
 80. Cordeiro, J.M., Nesterenko, V.V., Sicouri, S., Goodrow Jr, R.J., Treat, J.A., Desai, M., Wu, Y., Doss, M.X., Antzelevitch, C. and Di Diego, J.M., 2013. Identification and characterization of a transient outward K^+ current in human induced pluripotent stem cell-derived cardiomyocytes. *Journal of molecular and cellular cardiology*, 60, pp.36-46.
 81. Corden, B., Jarman, J., Whiffin, N., Tayal, U., Buchan, R., Sehmi, J., Harper, A., Midwinter, W., Lascelles, K., Markides, V. and Mason, M., 2019. Association of titin-truncating genetic variants with life-threatening cardiac arrhythmias in patients with dilated cardiomyopathy and implanted defibrillators. *JAMA Network Open*, 2(6), pp.e196520-e196520.

82. Corey, S. and Clapham, D.E., 1998. Identification of native atrial G-protein-regulated inwardly rectifying K⁺ (GIRK4) channel homomultimers. *Journal of Biological Chemistry*, 273(42), pp.27499-27504.
83. Corradi, D., Callegari, S., Maestri, R., Benussi, S. and Alfieri, O., 2008. Structural remodeling in atrial fibrillation. *Nature clinical practice Cardiovascular medicine*, 5(12), pp.782-796.
84. 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. and Alves, P.M., 2018. 3D aggregate culture improves metabolic maturation of human pluripotent stem cell derived cardiomyocytes. *Biotechnology and bioengineering*, 115(3), pp.630-644.
85. Courtemanche, M., Ramirez, R.J. and Nattel, S., 1998. Ionic mechanisms underlying human atrial action potential properties: insights from a mathematical model. *American Journal of Physiology-Heart and Circulatory Physiology*, 275(1), pp.H301-H321.
86. Crocini, C. and Gotthardt, M., 2021. Cardiac sarcomere mechanics in health and disease. *Biophysical reviews*, 13(5), pp.637-652.
87. Cumberland, M.J., Euchner, J., Azad, A.J., TN Vo, N., Kirchhof, P., Holmes, A.P., Denning, C. and Gehmlich, K., 2023. Generation of a human iPSC-derived cardiomyocyte/fibroblast engineered heart tissue model. *F1000Research*, 12, p.1224.
88. Cumberland, M.J., Riebel, L.L., Roy, A., O'Shea, C., Holmes, A.P., Denning, C., Kirchhof, P., Rodriguez, B. and Gehmlich, K., 2022. Basic research approaches to evaluate cardiac arrhythmia in heart failure and beyond. *Frontiers in Physiology*, 13, p.806366.
89. Cyganek, L., Tiburcy, M., Sekeres, K., Gerstenberg, K., Bohnenberger, H., Lenz, C., Henze, S., Stauske, M., Salinas, G., Zimmermann, W.H. and Hasenfuss, G., 2018. Deep phenotyping of human induced pluripotent stem cell-derived atrial and ventricular cardiomyocytes. *JCI insight*, 3(12).
90. Dasí, A., Roy, A., Sachetto, R., Camps, J., Bueno-Orovio, A. and Rodriguez, B., 2022. In-silico drug trials for precision medicine in atrial fibrillation: From ionic mechanisms to electrocardiogram-based predictions in structurally-healthy human atria. *Frontiers in physiology*, p.1927.
91. de Tombe, P.P., 2003. Cardiac myofilaments: mechanics and regulation. *Journal of biomechanics*, 36(5), pp.721-730.

92. Denham, N.C., Pearman, C.M., Caldwell, J.L., Madders, G.W., Eisner, D.A., Trafford, A.W. and Dibb, K.M., 2018. Calcium in the pathophysiology of atrial fibrillation and heart failure. *Frontiers in physiology*, 9, p.1380.
93. DeSilva, R.A., Graboys, T.B., Podrid, P.J. and Lown, B., 1980. Cardioversion and defibrillation. *American heart journal*, 100(6), pp.881-895.
94. Devalla, H.D., Schwach, V., Ford, J.W., Milnes, J.T., El-Haou, S., Jackson, C., Gkatzis, K., Elliott, D.A., Chuva de Sousa Lopes, S.M., Mummery, C.L. and Verkerk, A.O., 2015. Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO molecular medicine*, 7(4), pp.394-410.
95. Díez, J. and de Boer, R.A., 2022. Management of cardiac fibrosis is the largest unmet medical need in heart failure. *Cardiovascular research*, 118(2), pp.e20-e22.
96. DiFrancesco, D. and Borer, J.S., 2007. The funny current: cellular basis for the control of heart rate. *Drugs*, 67, pp.15-24.
97. Dohn, T.E., Ravisankar, P., Tirera, F.T., Martin, K.E., Gafranek, J.T., Duong, T.B., VanDyke, T.L., Touvron, M., Barske, L.A., Crump, J.G. and Waxman, J.S., 2019. Nr2f-dependent allocation of ventricular cardiomyocyte and pharyngeal muscle progenitors. *PLoS Genetics*, 15(2), p.e1007962.
98. Domínguez, F., Lalaguna, L., Martínez-Martín, I., Piqueras-Flores, J., Rasmussen, T.B., Zorio, E., Giovinazzo, G., Prados, B., Ochoa, J.P., Bornstein, B. and González-López, E., 2023. Titin Missense Variants as a Cause of Familial Dilated Cardiomyopathy. *Circulation*, 147(22), pp.1711-1713.
99. Doppler, S.A., Carvalho, C., Lahm, H., Deutsch, M.A., Dreßen, M., Puluca, N., Lange, R. and Krane, M., 2017. Cardiac fibroblasts: more than mechanical support. *Journal of thoracic disease*, 9(Suppl 1), p.S36.
100. Dorn, L.E., Petrosino, J.M., Wright, P. and Accornero, F., 2018. CTGF/CCN2 is an autocrine regulator of cardiac fibrosis. *Journal of molecular and cellular cardiology*, 121, pp.205-211.
101. Du, J., Xie, J., Zhang, Z., Tsujikawa, H., Fusco, D., Silverman, D., Liang, B. and Yue, L., 2010. TRPM7-mediated Ca²⁺ signals confer fibrogenesis in human atrial fibrillation. *Circulation research*, 106(5), pp.992-1003.
102. Eghbali, M., Czaja, M.J., Zeydel, M., Weiner, F.R., Zern, M.A., Seifter, S. and Blumenfeld, O.O., 1988. Collagen chain mRNAs in isolated heart cells from young and adult rats. *Journal of molecular and cellular cardiology*, 20(3), pp.267-276.

103. Eijgenraam, T.R., Silljé, H.H. and de Boer, R.A., 2020. Current understanding of fibrosis in genetic cardiomyopathies. *Trends in cardiovascular medicine*, 30(6), pp.353-361.
104. Elhamine, F., Radke, M.H., Pfitzer, G., Granzier, H., Gotthardt, M. and Stehle, R., 2014. Deletion of the titin N2B region accelerates myofibrillar force development but does not alter relaxation kinetics. *Journal of cell science*, 127(17), pp.3666-3674.
105. Ellinor, P.T., Lunetta, K.L., Glazer, N.L., Pfeufer, A., Alonso, A., Chung, M.K., Sinner, M.F., De Bakker, P.I., Mueller, M., Lubitz, S.A. and Fox, E., 2010. Common variants in KCNN3 are associated with lone atrial fibrillation. *Nature genetics*, 42(3), pp.240-244.
106. Elliott, P., Andersson, B., Arbustini, E., Bilinska, Z., Cecchi, F., Charron, P., Dubourg, O., Kühl, U., Maisch, B., McKenna, W.J. and Monserrat, L., 2008. Classification of the cardiomyopathies: a position statement from the European Society Of Cardiology Working Group on Myocardial and Pericardial Diseases. *European heart journal*, 29(2), pp.270-276.
107. Everett IV, T.H. and Olgin, J.E., 2007. Atrial fibrosis and the mechanisms of AF. *Heart rhythm*, 4(3), pp.S24-S27.
108. Everett, A.D., Heller, F. and Fisher, A., 1996. AT1Receptor Gene Regulation in Cardiac Myocytes and Fibroblasts. *Journal of molecular and cellular cardiology*, 28(8), pp.1727-1736.
109. Fabritz, L., Guasch, E., Antoniades, C., Bardinet, I., Benninger, G., Betts, T.R., Brand, E., Breithardt, G., Bucklar-Suchankova, G., Camm, A.J. and Cartlidge, D., 2016. Expert consensus document: defining the major health modifiers causing atrial fibrillation: a roadmap to underpin personalized prevention and treatment. *Nature reviews. Cardiology*, 13(4), p.230.
110. Fabrizio, L. and Regan, T.J., 1994. Alcoholic cardiomyopathy. *Cardiovascular drugs and therapy*, 8(1), pp.89-94.
111. Fan, H., Ma, L., Fan, B., Wu, J., Yang, Z. and Wang, L., 2014. Role of PDGFR- β /PI3K/AKT signaling pathway in PDGF-BB induced myocardial fibrosis in rats. *American journal of translational research*, 6(6), p.714.
112. Fang, L., Murphy, A.J. and Dart, A.M., 2017. A clinical perspective of anti-fibrotic therapies for cardiovascular disease. *Frontiers in pharmacology*, 8, p.186.
113. Fatkin, D., Huttner, I.G. and Johnson, R., 2019. Genetics of atrial cardiomyopathy. *Current opinion in cardiology*, 34(3), pp.275-281.

114. Fatkin, D., Seidman, C.E. and Seidman, J.G., 2014. Genetics and disease of ventricular muscle. *Cold Spring Harbor perspectives in medicine*, 4(1), p.a021063.
115. Feng, J., Armillei, M.K., Yu, A.S., Liang, B.T., Runnels, L.W. and Yue, L., 2019. Ca²⁺ signaling in cardiac fibroblasts and fibrosis-associated heart diseases. *Journal of cardiovascular development and disease*, 6(4), p.34.
116. Ferrer, A., Sebastián, R., Sánchez-Quintana, D., Rodriguez, J.F., Godoy, E.J., Martinez, L. and Saiz, J., 2015. Detailed anatomical and electrophysiological models of human atria and torso for the simulation of atrial activation. *PloS one*, 10(11), p.e0141573.
117. Feyen, D.A., McKeithan, W.L., Bruyneel, A.A., Spiering, S., Hörmann, L., Ulmer, B., Zhang, H., Briganti, F., Schweizer, M., Hegyi, B. and Liao, Z., 2020. Metabolic maturation media improve physiological function of human iPSC-derived cardiomyocytes. *Cell reports*, 32(3), p.107925.
118. Floy, M.E., Givens, S.E., Matthys, O.B., Mateyka, T.D., Kerr, C.M., Steinberg, A.B., Silva, A.C., Zhang, J., Mei, Y., Ogle, B.M. and McDevitt, T.C., 2021. Developmental lineage of human pluripotent stem cell-derived cardiac fibroblasts affects their functional phenotype. *The FASEB Journal*, 35(9), p.e21799.
119. Fomin, A., Gärtner, A., Cyganek, L., Tiburcy, M., Tuleta, I., Wellers, L., Folsche, L., Hobbach, A.J., von Frieling-Salewsky, M., Unger, A. and Hucke, A., 2021. Truncated titin proteins and titin haploinsufficiency are targets for functional recovery in human cardiomyopathy due to TTN mutations. *Science translational medicine*, 13(618), p.eabd3079.
120. Franaszczyk, M., Chmielewski, P., Truszkowska, G., Stawinski, P., Michalak, E., Rydzanicz, M., Sobieszczanska-Malek, M., Pollak, A., Szczygiał, J., Kosinska, J. and Parulski, A., 2017. Titin truncating variants in dilated cardiomyopathy—prevalence and genotype-phenotype correlations. *PLoS One*, 12(1), p.e0169007.
121. Frangogiannis, N.G., 2019. Cardiac fibrosis: cell biological mechanisms, molecular pathways and therapeutic opportunities. *Molecular aspects of medicine*, 65, pp.70-99.
122. Frangogiannis, N.G., 2021. Cardiac fibrosis. *Cardiovascular research*, 117(6), pp.1450-1488.
123. Freiburg, A. and Gautel, M., 1996. A Molecular Map of the Interactions between Titin and Myosin-Binding Protein C: Implications for Sarcomeric Assembly in Familial Hypertrophic Cardiomyopathy. *European Journal of Biochemistry*, 235(1-2), pp.317-323.

124. Freichel, M., Berlin, M., Schürger, A., Mathar, I., Bacmeister, L., Medert, R., Frede, W., Marx, A., Segin, S. and Londoño, J.E.C., 2017. TRP Channels in the Heart. *Neurobiology of TRP channels*, 1, pp.149-86.
125. Friberg, L. and Bergfeldt, L., 2013. Atrial fibrillation prevalence revisited. *Journal of internal medicine*, 274(5), pp.461-468.
126. Furst, D.O., Nave, R., Osborn, M. and Weber, K., 1989. Repetitive titin epitopes with a 42 nm spacing coincide in relative position with known A band striations also identified by major myosin-associated proteins. An immunoelectron-microscopical study on myofibrils. *Journal of cell science*, 94(1), pp.119-125.
127. Fürst, D.O., Osborn, M., Nave, R. and Weber, K., 1988. The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *The Journal of cell biology*, 106(5), pp.1563-1572.
128. Fye, W.B., 1994. A history of the origin, evolution, and impact of electrocardiography. *The American journal of cardiology*, 73(13), pp.937-949.
129. Gaidai, O., Cao, Y. and Loginov, S., 2023. Global cardiovascular diseases death rate prediction. *Current Problems in Cardiology*, p.101622.
130. Gallini, R., Lindblom, P., Bondjers, C., Betsholtz, C. and Andrae, J., 2016. PDGF-A and PDGF-B induces cardiac fibrosis in transgenic mice. *Experimental cell research*, 349(2), pp.282-290.
131. Ganesh, S.K., Arnett, D.K., Assimes, T.L., Basson, C.T., Chakravarti, A., Ellinor, P.T., Engler, M.B., Goldmuntz, E., Herrington, D.M., Hershberger, R.E. and Hong, Y., 2013. Genetics and genomics for the prevention and treatment of cardiovascular disease: update: a scientific statement from the American Heart Association. *Circulation*, 128(25), pp.2813-2851.
132. Gao, Q.Y., Zhang, H.F., Chen, Z.T., Li, Y.W., Wang, S.H., Wen, Z.Z., Xie, Y., Mai, J.T., Wang, J.F. and Chen, Y.X., 2021. Construction and Analysis of a ceRNA Network in Cardiac Fibroblast During Fibrosis Based on in vivo and in vitro Data. *Frontiers in Genetics*, 11, p.503256.
133. Garg, P., Garg, V., Shrestha, R., Sanguinetti, M.C., Kamp, T.J. and Wu, J.C., 2018. Human induced pluripotent stem cell–derived cardiomyocytes as models for cardiac channelopathies: a primer for non-electrophysiologists. *Circulation research*, 123(2), pp.224-243.

134. Gassanov, N., Er, F., Zagidullin, N., Jankowski, M., Gutkowska, J. and Hoppe, U.C., 2008. Retinoid acid-induced effects on atrial and pacemaker cell differentiation and expression of cardiac ion channels. *Differentiation*, 76(9), pp.971-980.
135. Gaziano, T., Reddy, K.S., Paccaud, F., Horton, S. and Chaturvedi, V., 2006. Cardiovascular disease. *Disease Control Priorities in Developing Countries. 2nd edition*.
136. Ge, S.X., Son, E.W. and Yao, R., 2018. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. *BMC bioinformatics*, 19(1), pp.1-24.
137. Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitás, K., Sasse-Klaassen, S., Seidman, J.G., Seidman, C., Granzier, H., Labeit, S. and Frenneaux, M., 2002. Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nature genetics*, 30(2), pp.201-204.
138. Geselowitz, D.B., 1989. On the theory of the electrocardiogram. *Proceedings of the IEEE*, 77(6), pp.857-876.
139. Gilles, G., McCulloch, A.D., Brakebusch, C.H. and Herum, K.M., 2020. Maintaining resting cardiac fibroblasts in vitro by disrupting mechanotransduction. *PLoS One*, 15(10), p.e0241390.
140. Gintant, G., Burridge, P., Gepstein, L., Harding, S., Herron, T., Hong, C., Jalife, J., Wu, J.C. and American Heart Association Council on Basic Cardiovascular Sciences, 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), pp.e75-e92.
141. Glitsch, H.G., 2001. Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiological reviews*, 81(4), pp.1791-1826.
142. Goedel, A., My, I., Sinnecker, D. and Moretti, A., 2017. Perspectives and challenges of pluripotent stem cells in cardiac arrhythmia research. *Current cardiology reports*, 19(3), p.23.
143. Goktas Sahoglu, S., Kazci, Y.E., Tuncay, E., Torun, T., Akdeniz, C., Tuzcu, V. and Cagavi, E., 2022. Functional evaluation of the tachycardia patient-derived iPSC cardiomyocytes carrying a novel pathogenic SCN5A variant. *Journal of Cellular Physiology*, 237(10), pp.3900-3911.
144. Gomez-Outes, A., Suárez-Gea, M.L. and García-Pinilla, J.M., 2017. Causes of death in atrial fibrillation: Challenges and opportunities. *Trends in Cardiovascular Medicine*, 27(7), pp.494-503.

145. Goodyer, W.R., Dunn, K., Caleshu, C., Jackson, M., Wylie, J., Moscarello, T., Platt, J., Reuter, C., Smith, A., Trela, A. and Ceresnak, S.R., 2019. Broad genetic testing in a clinical setting uncovers a high prevalence of titin loss-of-function variants in very early onset atrial fibrillation. *Circulation: Genomic and Precision Medicine*, 12(11), p.e002713.
146. Goversen, B., van der Heyden, M.A., van Veen, T.A. and de Boer, T.P., 2018. The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on IK1. *Pharmacology & therapeutics*, 183, pp.127-136.
147. Gramlich, M., Michely, B., Krohne, C., Heuser, A., Erdmann, B., Klaassen, S., Hudson, B., Magarin, M., Kirchner, F., Todiras, M. and Granzier, H., 2009. Stress-induced dilated cardiomyopathy in a knock-in mouse model mimicking human titin-based disease. *Journal of molecular and cellular cardiology*, 47(3), pp.352-358.
148. Grandi, E., Pandit, S.V., Voigt, N., Workman, A.J., Dobrev, D., Jalife, J. and Bers, D.M., 2011. Human atrial action potential and Ca²⁺ model: sinus rhythm and chronic atrial fibrillation. *Circulation research*, 109(9), pp.1055-1066.
149. Granzier, H. and Labeit, S., 2007. Structure–function relations of the giant elastic protein titin in striated and smooth muscle cells. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, 36(6), pp.740-755.
150. Granzier, H.L., Hutchinson, K.R., Tonino, P., Methawasin, M., Li, F.W., Slater, R.E., Bull, M.M., Saripalli, C., Pappas, C.T., Gregorio, C.C. and Smith, J.E., 2014. Deleting titin's I-band/A-band junction reveals critical roles for titin in biomechanical sensing and cardiac function. *Proceedings of the National Academy of Sciences*, 111(40), pp.14589-14594.
151. Gray, B. and Behr, E.R., 2016. New insights into the genetic basis of inherited arrhythmia syndromes. *Circulation: Cardiovascular Genetics*, 9(6), pp.569-577.
152. Grogan, M., Redfield, M.M., Bailey, K.R., Reeder, G.S., Gersh, B.J., Edwards, W.D. and Rodeheffer, R.J., 1995. Long-term outcome of patients with biopsy-proved myocarditis: comparison with idiopathic dilated cardiomyopathy. *Journal of the American College of Cardiology*, 26(1), pp.80-84.
153. Gudbjartsson, D.F., Arnar, D.O., Helgadóttir, A., Gretarsdóttir, S., Holm, H., Sigurdsson, A., Jonasdóttir, A., Baker, A., Thorleifsson, G., Kristjansson, K. and Palsson, A., 2007. Variants conferring risk of atrial fibrillation on chromosome 4q25. *Nature*, 448(7151), pp.353-357.

154. Guinamard, R., Chatelier, A., Demion, M., Potreau, D., Patri, S., Rahmati, M. and Bois, P., 2004. Functional characterization of a Ca²⁺-activated non-selective cation channel in human atrial cardiomyocytes. *The Journal of physiology*, 558(1), pp.75-83.
155. Guo, W., Schafer, S., Greaser, M.L., Radke, M.H., Liss, M., Govindarajan, T., Maatz, H., Schulz, H., Li, S., Parrish, A.M. and Dauksaite, V., 2012. RBM20, a gene for hereditary cardiomyopathy, regulates titin splicing. *Nature medicine*, 18(5), pp.766-773.
156. Gurantz, D., Cowling, R.T., Varki, N., Frikovsky, E., Moore, C.D. and Greenberg, B.H., 2005. IL-1 β and TNF- α upregulate angiotensin II type 1 (AT1) receptors on cardiac fibroblasts and are associated with increased AT1 density in the post-MI heart. *Journal of molecular and cellular cardiology*, 38(3), pp.505-515.
157. Gwanyanya, A. and Mubagwa, K., 2022. Emerging role of transient receptor potential (TRP) ion channels in cardiac fibroblast pathophysiology. *Frontiers in Physiology*, 13, p.968393.
158. Haas, J., Frese, K.S., Peil, B., Kloos, W., Keller, A., Nietsch, R., Feng, Z., Müller, S., Kayvanpour, E., Vogel, B. and Sedaghat-Hamedani, F., 2015. Atlas of the clinical genetics of human dilated cardiomyopathy. *European heart journal*, 36(18), pp.1123-1135.
159. Hackman, P., Vihola, A., Haravuori, H., Marchand, S., Sarparanta, J., de Seze, J., Labeit, S., Witt, C., Peltonen, L., Richard, I. and Udd, B., 2002. Tibial muscular dystrophy is a titinopathy caused by mutations in TTN, the gene encoding the giant skeletal-muscle protein titin. *The American Journal of Human Genetics*, 71(3), pp.492-500.
160. Haggerty, C.M., Damrauer, S.M., Levin, M.G., Birtwell, D., Carey, D.J., Golden, A.M., Hartzel, D.N., Hu, Y., Judy, R., Kelly, M.A. and Kember, R.L., 2019. Genomics-first evaluation of heart disease associated with titin-truncating variants. *Circulation*, 140(1), pp.42-54.
161. Hall, C., Law, J.P., Reyat, J.S., Cumberland, M.J., Hang, S., Vo, N.T., Raniga, K., Weston, C.J., O'Shea, C., Townsend, J.N. and Gehmlich, K., 2023. Chronic activation of human cardiac fibroblasts in vitro attenuates the reversibility of the myofibroblast phenotype. *Scientific Reports*, 13(1), p.12137.
162. Hanna, A., Humeres, C. and Frangogiannis, N.G., 2021. The role of Smad signaling cascades in cardiac fibrosis. *Cellular signalling*, 77, p.109826.
163. Haravuori, H., Vihola, A., Straub, V., Auranen, M., Richard, I., Marchand, S., Voit, T., Labeit, S., Somer, H., Peltonen, L. and Beckmann, J.S., 2001. Secondary calpain3

- deficiency in 2q-linked muscular dystrophy: titin is the candidate gene. *Neurology*, 56(7), pp.869-877.
164. Harikrishnan, S., Jeemon, P., Mini, G.K., Thankappan, K.R. and Sylaja, P., 2018. GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017.
 165. Henkens, M.T., Raafs, A.G., Vanloon, T., Vos, J.L., Vandenwijngaard, A., Brunner, H.G., Krapels, I.P., Knackstedt, C., Gerretsen, S., Hazebroek, M.R. and Vernooij, K., 2023. Left Atrial Function in Patients with Titin Cardiomyopathy. *Journal of Cardiac Failure*.
 166. Heras-Bautista, C.O., Mikhael, N., Lam, J., Shinde, V., Katsen-Globa, A., Dieluweit, S., Molcanyi, M., Uvarov, V., Jütten, P., Sahito, R.G. and Mederos-Henry, F., 2019. Cardiomyocytes facing fibrotic conditions re-express extracellular matrix transcripts. *Acta biomaterialia*, 89, pp.180-192.
 167. Herman, D.S., Lam, L., Taylor, M.R., Wang, L., Teekakirikul, P., Christodoulou, D., Conner, L., DePalma, S.R., McDonough, B., Sparks, E. and Teodorescu, D.L., 2012. Truncations of titin causing dilated cardiomyopathy. *New England Journal of Medicine*, 366(7), pp.619-628.
 168. Hershberger, R.E., Hedges, D.J. and Morales, A., 2013. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. *Nature reviews cardiology*, 10(9), pp.531-547.
 169. Herum, K.M., Lunde, I.G., Skrbic, B., Florholmen, G., Behmen, D., Sjaastad, I., Carlson, C.R., Gomez, M.F. and Christensen, G., 2013. Syndecan-4 signaling via NFAT regulates extracellular matrix production and cardiac myofibroblast differentiation in response to mechanical stress. *Journal of molecular and cellular cardiology*, 54, pp.73-81.
 170. Hinderer, S. and Schenke-Layland, K., 2019. Cardiac fibrosis—A short review of causes and therapeutic strategies. *Advanced drug delivery reviews*, 146, pp.77-82.
 171. Hindricks, G., Potpara, T., Dagres, N., Arbelo, E., Bax, J.J., Blomström-Lundqvist, C., Boriani, G., Castella, M., Dan, G.A., Dilaveris, P.E. and Fauchier, L., 2021. 2020 ESC Guidelines for the diagnosis and management of atrial fibrillation developed in collaboration with the European Association for Cardio-Thoracic Surgery (EACTS) The Task Force for the diagnosis and management of atrial fibrillation of the European Society of Cardiology (ESC) Developed with the special contribution of the European

- Heart Rhythm Association (EHRA) of the ESC. *European heart journal*, 42(5), pp.373-498.
172. Hinson, J.T., Chopra, A., Nafissi, N., Polacheck, W.J., Benson, C.C., Swist, S., Gorham, J., Yang, L., Schafer, S., Sheng, C.C. and Haghighi, A., 2015. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science*, 349(6251), pp.982-986.
 173. Holmes, A.P., Saxena, P., Kabir, S.N., O'Shea, C., Kuhlmann, S.M., Gupta, S., Fobian, D., Apicella, C., O'Reilly, M., Syeda, F. and Reyat, J.S., 2021. Atrial resting membrane potential confers sodium current sensitivity to propafenone, flecainide and dronedarone. *Heart Rhythm*, 18(7), pp.1212-1220.
 174. Holmes, A.P., Saxena, P., Kabir, S.N., O'Shea, C., Kuhlmann, S.M., Gupta, S., Fobian, D., Apicella, C., O'Reilly, M., Syeda, F. and Reyat, J.S., 2021. Atrial resting membrane potential confers sodium current sensitivity to propafenone, flecainide and dronedarone. *Heart Rhythm*, 18(7), pp.1212-1220.
 175. Hoorntje, E.T., van Spaendonck-Zwarts, K.Y., Te Rijdt, W.P., Boven, L., Vink, A., van der Smagt, J.J., Asselbergs, F.W., van Wijngaarden, J., Hennekam, E.A., Pinto, Y.M. and Deprez, R.H.L., 2018. The first titin (c. 59926+ 1G> A) founder mutation associated with dilated cardiomyopathy. *European journal of heart failure*, 20(4), p.803.
 176. Horowitz, R., Kempner, E.S., Bisher, M.E. and Podolsky, R.J., 1986. A physiological role for titin and nebulin in skeletal muscle. *Nature*, 323(6084), pp.160-164.
 177. Huang, G., Bisaria, A., Wakefield, D.L., Yamawaki, T.M., Luo, X., Zhang, J.A., Vigneault, P., Wang, J., Reagan, J.D., Oliverio, O. and Zhou, H., 2023. Titin-truncating variants in iPSC cardiomyocytes induce pathogenic proteinopathy and sarcomere defects with preserved core contractile machinery. *Stem Cell Reports*, 18(1), pp.220-236.
 178. Huang, S., Chen, B., Humeres, C., Alex, L., Hanna, A. and Frangogiannis, N.G., 2020. The role of Smad2 and Smad3 in regulating homeostatic functions of fibroblasts in vitro and in adult mice. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1867(7), p.118703.
 179. Huttner, I.G., Wang, L.W., Santiago, C.F., Horvat, C., Johnson, R., Cheng, D., von Frieling-Salewsky, M., Hillcoat, K., Bemand, T.J., Trivedi, G. and Braet, F., 2018. A-band titin truncation in zebrafish causes dilated cardiomyopathy and hemodynamic stress intolerance. *Circulation: Genomic and Precision Medicine*, 11(8), p.e002135.
 180. Ihara, K., Sasano, T., Hiraoka, Y., Togo-Ohno, M., Soejima, Y., Sawabe, M., Tsuchiya, M., Ogawa, H., Furukawa, T. and Kuroyanagi, H., 2020. A missense mutation

- in the RSRSP stretch of Rbm20 causes dilated cardiomyopathy and atrial fibrillation in mice. *Scientific reports*, 10(1), pp.1-14.
181. Iles, L., Pflugner, H., Lefkovits, L., Butler, M.J., Kistler, P.M., Kaye, D.M. and Taylor, A.J., 2011. Myocardial fibrosis predicts appropriate device therapy in patients with implantable cardioverter-defibrillators for primary prevention of sudden cardiac death. *Journal of the American College of Cardiology*, 57(7), pp.821-828.
 182. Imaeda, A., Tanaka, S., Tonegawa, K., Fuchigami, S., Obana, M., Maeda, M., Kihara, M., Kiyonari, H., Conway, S.J., Fujio, Y. and Nakayama, H., 2019. Myofibroblast β 2 adrenergic signaling amplifies cardiac hypertrophy in mice. *Biochemical and biophysical research communications*, 510(1), pp.149-155.
 183. Ivey, M.J. and Tallquist, M.D., 2016. Defining the cardiac fibroblast. *Circulation Journal*, 80(11), pp.2269-2276.
 184. Jahns, R., Boivin, V., Hein, L., Triebel, S., Angermann, C.E., Ertl, G. and Lohse, M.J., 2004. Direct evidence for a β 1-adrenergic receptor-directed autoimmune attack as a cause of idiopathic dilated cardiomyopathy. *The Journal of clinical investigation*, 113(10), pp.1419-1429.
 185. Jakob, D., Klesen, A., Allegrini, B., Darkow, E., Aria, D., Emig, R., Chica, A.S., Rog-Zielinska, E.A., Guth, T., Beyersdorf, F. and Kari, F.A., 2021. Piezo1 and BKCa channels in human atrial fibroblasts: Interplay and remodelling in atrial fibrillation. *Journal of Molecular and Cellular Cardiology*, 158, pp.49-62.
 186. Jankowich, M. and Choudhary, G., 2020. Endothelin-1 levels and cardiovascular events. *Trends in cardiovascular medicine*, 30(1), pp.1-8.
 187. Janse, M.J. and Wit, A.L., 1989. Electrophysiological mechanisms of ventricular arrhythmias resulting from myocardial ischemia and infarction. *Physiological reviews*, 69(4), pp.1049-1169.
 188. Jansweijer, J.A., Nieuwhof, K., Russo, F., Hoorntje, E.T., Jongbloed, J.D., Lekanke Deprez, R.H., Postma, A.V., Bronk, M., van Rijsingen, I.A., de Haij, S. and Biagini, E., 2017. Truncating titin mutations are associated with a mild and treatable form of dilated cardiomyopathy. *European journal of heart failure*, 19(4), pp.512-521.
 189. Jeevaratnam, K., Chadda, K.R., Huang, C.L.H. and Camm, A.J., 2018. Cardiac potassium channels: physiological insights for targeted therapy. *Journal of cardiovascular pharmacology and therapeutics*, 23(2), pp.119-129.
 190. Jewell, J.L. and Guan, K.L., 2013. Nutrient signaling to mTOR and cell growth. *Trends in biochemical sciences*, 38(5), pp.233-242.

191. Jeziorowska, D., Fontaine, V., Jouve, C., Villard, E., Dussaud, S., Akbar, D., Letang, V., Cervello, P., Itier, J.M., Pruniaux, M.P. and Hulot, J.S., 2017. Differential sarcomere and electrophysiological maturation of human iPSC-derived cardiac myocytes in monolayer vs. aggregation-based differentiation protocols. *International journal of molecular sciences*, 18(6), p.1173.
192. Jiang, Z., Zhong, G., Wen, L., Hong, Y., Fang, S., Sun, P., Li, S., Li, S. and Feng, G., 2016. The role of platelet-derived growth factor-B/platelet-derived growth factor receptor- β signaling in chronic AF. *Cardiology*, 133(4), pp.242-256.
193. Jimenez-Vazquez, E.N., Jain, A. and Jones, D.K., 2022. Enhancing iPSC-CM Maturation Using a Matrigel-Coated Micropatterned PDMS Substrate. *Current Protocols*, 2(11), p.e601.
194. Joukar, S., 2021. A comparative review on heart ion channels, action potentials and electrocardiogram in rodents and human: extrapolation of experimental insights to clinic. *Laboratory Animal Research*, 37(1), pp.1-15.
195. Jung, P., Seibert, F., Fakuade, F.E., Ignatyeva, N., Sampathkumar, S., Ritter, M., Li, H., Mason, F.E., Ebert, A. and Voigt, N., 2022. Increased cytosolic calcium buffering contributes to a cellular arrhythmogenic substrate in iPSC-cardiomyocytes from patients with dilated cardiomyopathy. *Basic research in cardiology*, 117(1), pp.1-18.
196. Kalstoe, S.M., Mattingsdal, M., Rypdal, K.B., Frisk, M., Lundegaard, P.R., Tveit, A., Olesen, M.S., Lunde, I.G. and Christophersen, I.E., 2023. Atrial Sarcomeric Dysfunction in Tntv Mice Indicates a Causal Relationship Between Rare Variation in TTN and Early Onset Atrial Fibrillation in Patients. *Circulation*, 148(Suppl_1), pp.A15046-A15046.
197. Kamel, H., Okin, P.M., Elkind, M.S. and Iadecola, C., 2016. Atrial fibrillation and mechanisms of stroke: time for a new model. *Stroke*, 47(3), pp.895-900.
198. Kane, C., Du, D.T., Hellen, N. and Terracciano, C.M., 2016. The Fallacy of Assigning Chamber Specificity to iPSC Cardiac Myocytes from Action Potential Morphology. *Biophysical Journal*, 110(1), p.281.
199. Kaur, K., Zarzoso, M., Ponce-Balbuena, D., Guerrero-Serna, G., Hou, L., Musa, H. and Jalife, J., 2013. TGF- β 1, released by myofibroblasts, differentially regulates transcription and function of sodium and potassium channels in adult rat ventricular myocytes. *PloS one*, 8(2), p.e55391.
200. Kay, G.N. and Shepard, R.B., 2017. Stimulation and excitation of cardiac tissues. *Clinical Cardiac Pacing, Defibrillation and Resynchronization Therapy*, pp.61-113.

201. Ke, X., Guo, W., Peng, Y., Feng, Z., Huang, Y.T., Deng, M., Wei, M.X. and Wang, Z.X., 2022. Investigation into the role of *Stmn2* in vascular smooth muscle phenotype transformation during vascular injury via RNA sequencing and experimental validation. *Environmental Science and Pollution Research*, 29(3), pp.3498-3509.
202. Kellermayer, D., Tordai, H., Kiss, B., Török, G., Péter, D.M., Sayour, A.A., Pólos, M., Hartyánszky, I., Szilveszter, B., Labeit, S. and Gángó, A., 2023. Truncated titin is structurally integrated into the human dilated cardiomyopathic sarcomere. *The Journal of Clinical Investigation*.
203. Kellermayer, D., Tordai, H., Kiss, B., Török, G., Sayour, A.A., Pólos, M., Hartyánszky, I., Szilveszter, B., Labeit, S., Gángó, A. and Bedics, G., 2023. Truncated titin is integrated into the human dilated cardiomyopathic sarcomere. *Biophysical Journal*, 122(3), p.454a.
204. Khan, R., 2004. Identifying and understanding the role of pulmonary vein activity in atrial fibrillation. *Cardiovascular research*, 64(3), pp.387-394.
205. Kiès, P., Bootsma, M., Bax, J., Schalij, M.J. and van der Wall, E.E., 2006. Arrhythmogenic right ventricular dysplasia/cardiomyopathy: screening, diagnosis, and treatment. *Heart Rhythm*, 3(2), pp.225-234.
206. Kim, C., Majdi, M., Xia, P., Wei, K.A., Talantova, M., Spiering, S., Nelson, B., Mercola, M. and Chen, H.S.V., 2010. Non-cardiomyocytes influence the electrophysiological maturation of human embryonic stem cell-derived cardiomyocytes during differentiation. *Stem cells and development*, 19(6), pp.783-795.
207. Kirchhof, P., 2017. The future of atrial fibrillation management: integrated care and stratified therapy. *The Lancet*, 390(10105), pp.1873-1887.
208. Kirk, R., Edwards, L.B., Aurora, P., Taylor, D.O., Christie, J., Dobbels, F., Kucheryavaya, A.Y., Rahmel, A.O. and Hertz, M.I., 2008. Registry of the International Society for Heart and Lung Transplantation: eleventh official pediatric heart transplantation report—2008. *The Journal of heart and lung transplantation*, 27(9), pp.970-977.
209. Kneller, J., Zou, R., Vigmond, E.J., Wang, Z., Leon, L.J. and Nattel, S., 2002. Cholinergic atrial fibrillation in a computer model of a two-dimensional sheet of canine atrial cells with realistic ionic properties. *Circulation research*, 90(9), pp.e73-e87.
210. Knollmann, B.C., 2013. Induced pluripotent stem cell-derived cardiomyocytes: Boutique science or valuable arrhythmia model?. *Circulation research*, 112(6), pp.969-976.

211. Kofron, C.M. and Mende, U., 2017. In vitro models of the cardiac microenvironment to study myocyte and non-myocyte crosstalk: bioinspired approaches beyond the polystyrene dish. *The Journal of physiology*, 595(12), pp.3891-3905.
212. Kolossov, E., Lu, Z., Drobinskaya, I., Gassanov, N., Duan, Y., Sauer, H., Manzke, O., Bloch, W., Bohlen, H., Hescheler, J. and Fleischmann, B.K., 2005. Identification and characterization of embryonic stem cell-derived pacemaker and atrial cardiomyocytes. *The FASEB journal*, 19(6), pp.1-25.
213. Kong, P., Christia, P. and Frangogiannis, N.G., 2014. The pathogenesis of cardiac fibrosis. *Cellular and molecular life sciences*, 71, pp.549-574.
214. Kornreich, B.G., 2007. The patch clamp technique: principles and technical considerations. *Journal of Veterinary Cardiology*, 9(1), pp.25-37.
215. Kötter, S., Andresen, C. and Krüger, M., 2014. Titin: central player of hypertrophic signaling and sarcomeric protein quality control. *Biological Chemistry*, 395(11), pp.1341-1352.
216. Kramer, J.W., Post, M.A., Brown, A.M. and Kirsch, G.E., 1998. Modulation of potassium channel gating by coexpression of Kv2. 1 with regulatory Kv5. 1 or Kv6. 1 α -subunits. *American Journal of Physiology-Cell Physiology*, 274(6), pp.C1501-C1510.
217. Krapivinsky, G., Gordon, E.A., Wickman, K., Velimirović, B., Krapivinsky, L. and Clapham, D.E., 1995. The G-protein-gated atrial K⁺ channel IK_{ACh} is a heteromultimer of two inwardly rectifying K⁺-channel proteins. *Nature*, 374(6518), pp.135-141.
218. Krenning, G., Zeisberg, E.M. and Kalluri, R., 2010. The origin of fibroblasts and mechanism of cardiac fibrosis. *Journal of cellular physiology*, 225(3), pp.631-637.
219. Krijthe, B.P., Kunst, A., Benjamin, E.J., Lip, G.Y., Franco, O.H., Hofman, A., Witteman, J.C., Stricker, B.H. and Heeringa, J., 2013. Projections on the number of individuals with atrial fibrillation in the European Union, from 2000 to 2060. *European heart journal*, 34(35), pp.2746-2751.
220. Krüger, M., Kötter, S., Grützner, A., Lang, P., Andresen, C., Redfield, M.M., Butt, E., Dos Remedios, C.G. and Linke, W.A., 2009. Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circulation research*, 104(1), pp.87-94.
221. Kubalak, S.W., Miller-Hance, W.C., O'Brien, T.X., Dyson, E. and Chien, K.R., 1994. Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *Journal of Biological Chemistry*, 269(24), pp.16961-16970.

222. Kurachi, Y., 1995. G protein regulation of cardiac muscarinic potassium channel. *The American journal of physiology*, 269(4 Pt 1), pp.C821-C830.
223. Labeit, S., Kolmerer, B. and Linke, W.A., 1997. The giant protein titin: emerging roles in physiology and pathophysiology. *Circulation Research*, 80(2), pp.290-294.
224. Landry, N.M., Rattan, S.G. and Dixon, I.M., 2019. An improved method of maintaining primary murine cardiac fibroblasts in two-dimensional cell culture. *Scientific reports*, 9(1), p.12889.
225. Lane, D.A., Skjøth, F., Lip, G.Y., Larsen, T.B. and Kotecha, D., 2017. Temporal trends in incidence, prevalence, and mortality of atrial fibrillation in primary care. *Journal of the American Heart Association*, 6(5), p.e005155.
226. Lange, S., Xiang, F., Yakovenko, A., Vihola, A., Hackman, P., Rostkova, E., Kristensen, J., Brandmeier, B., Franzen, G., Hedberg, B. and Gunnarsson, L.G., 2005. The kinase domain of titin controls muscle gene expression and protein turnover. *Science*, 308(5728), pp.1599-1603.
227. Lau, D.H., Nattel, S., Kalman, J.M. and Sanders, P., 2017. Modifiable risk factors and atrial fibrillation. *Circulation*, 136(6), pp.583-596.
228. Lau, D.H., Shipp, N.J., Kelly, D.J., Thanigaimani, S., Neo, M., Kuklik, P., Lim, H.S., Zhang, Y., Drury, K., Wong, C.X. and Chia, N.H., 2013. Atrial arrhythmia in ageing spontaneously hypertensive rats: unraveling the substrate in hypertension and ageing. *PloS one*, 8(8), p.e72416.
229. Law, B.A. and Carver, W.E., 2013. Activation of cardiac fibroblasts by ethanol is blocked by TGF- β inhibition. *Alcoholism: Clinical and Experimental Research*, 37(8), pp.1286-1294.
230. Leask, A., 2010. Potential therapeutic targets for cardiac fibrosis: TGF β , angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circulation research*, 106(11), pp.1675-1680.
231. Lee, J., Termglinchan, V., Diecke, S., Itzhaki, I., Lam, C.K., Garg, P., Lau, E., Greenhaw, M., Seeger, T., Wu, H. and Zhang, J.Z., 2019. Activation of PDGF pathway links LMNA mutation to dilated cardiomyopathy. *Nature*, 572(7769), pp.335-340.
232. Lei, M., Wu, L., Terrar, D.A. and Huang, C.L.H., 2018. Modernized classification of cardiac antiarrhythmic drugs. *Circulation*, 138(17), pp.1879-1896.
233. Lemcke, H., Skorska, A., Lang, C.I., Johann, L. and David, R., 2020. Quantitative evaluation of the sarcomere network of human iPSC-derived cardiomyocytes using

- single-molecule localization microscopy. *International journal of molecular sciences*, 21(8), p.2819.
234. Lemoine, M.D., Mannhardt, I., Breckwoldt, K., Prondzynski, M., Flenner, F., Ulmer, B., Hirt, M.N., Neuber, C., Horváth, A., Kloth, B. and Reichenspurner, H., 2017. Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density. *Scientific reports*, 7(1), pp.1-11.
 235. Lewandowski, J., Rozwadowska, N., Kolanowski, T.J., Malcher, A., Zimna, A., Rugowska, A., Fiedorowicz, K., Łabędź, W., Kubaszewski, Ł., Chojnacka, K. and Bednarek-Rajewska, K., 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), pp.1047-1067.
 236. Li, G.R., Sun, H.Y., Chen, J.B., Zhou, Y., Tse, H.F. and Lau, C.P., 2009. Characterization of multiple ion channels in cultured human cardiac fibroblasts. *PLoS one*, 4(10), p.e7307.
 237. Liao, D., Chen, W., Tan, C.Y., Wong, J.X., Chan, P.S., Tan, L.W., Foo, R. and Jiang, J., 2019. Upregulation of Yy1 suppresses dilated cardiomyopathy caused by Ttn insufficiency. *Scientific Reports*, 9(1), p.16330.
 238. Lin, B., Li, Y., Han, L., Kaplan, A.D., Ao, Y., Kalra, S., Bett, G.C., Rasmusson, R.L., Denning, C. and Yang, L., 2015. Modeling and study of the mechanism of dilated cardiomyopathy using induced pluripotent stem cells derived from individuals with Duchenne muscular dystrophy. *Disease Models & Mechanisms*, 8(5), pp.457-466.
 239. Linneberg, C., Toft, C.L.F., Kjaer-Sorensen, K. and Laursen, L.S., 2019. L1cam-mediated developmental processes of the nervous system are differentially regulated by proteolytic processing. *Scientific reports*, 9(1), p.3716.
 240. Linz, D., Elliott, A.D., Hohl, M., Malik, V., Schotten, U., Dobrev, D., Nattel, S., Böhm, M., Floras, J., Lau, D.H. and Sanders, P., 2019. Role of autonomic nervous system in atrial fibrillation. *International Journal of Cardiology*, 287, pp.181-188.
 241. Lip, G.Y. and Lane, D.A., 2015. Stroke prevention in atrial fibrillation: a systematic review. *Jama*, 313(19), pp.1950-1962.
 242. Litviňuková, M., Talavera-López, C., Maatz, H., Reichart, D., Worth, C.L., Lindberg, E.L., Kanda, M., Polanski, K., Heinig, M., Lee, M. and Nadelmann, E.R., 2020. Cells of the adult human heart. *Nature*, 588(7838), pp.466-472.
 243. Luk, A., Ahn, E., Soor, G.S. and Butany, J., 2009. Dilated cardiomyopathy: a review. *Journal of clinical pathology*, 62(3), pp.219-225.

244. Luu, M., Stevenson, W.G., Stevenson, L.W., Baron, K. and Walden, J., 1989. Diverse mechanisms of unexpected cardiac arrest in advanced heart failure. *Circulation*, 80(6), pp.1675-1680.
245. Ma, J., Guo, L., Fiene, S.J., Anson, B.D., Thomson, J.A., Kamp, T.J., Kolaja, K.L., Swanson, B.J. and 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), pp.H2006-H2017.
246. Ma, N., Zhang, J.Z., Itzhaki, I., Zhang, S.L., Chen, H., Haddad, F., Kitani, T., Wilson, K.D., Tian, L., Shrestha, R. and Wu, H., 2018. Determining the pathogenicity of a genomic variant of uncertain significance using CRISPR/Cas9 and human-induced pluripotent stem cells. *Circulation*, 138(23), pp.2666-2681.
247. Ma, Y.H., Ling, S. and Ives, H.E., 1999. Mechanical strain increases PDGF-B and PDGF β receptor expression in vascular smooth muscle cells. *Biochemical and biophysical research communications*, 265(2), pp.606-610.
248. Ma, Z.G., Yuan, Y.P., Wu, H.M., Zhang, X. and Tang, Q.Z., 2018. Cardiac fibrosis: new insights into the pathogenesis. *International journal of biological sciences*, 14(12), p.1645.
249. Machiraju, P. and Greenway, S.C., 2019. Current methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes. *World journal of stem cells*, 11(1), p.33.
250. Maisel, W.H. and Stevenson, L.W., 2003. AF in heart failure: epidemiology, pathophysiology, and rationale for therapy. *The American journal of cardiology*, 91(6), pp.2-8.
251. Makary, S., Voigt, N., Maguy, A., Wakili, R., Nishida, K., Harada, M., Dobrev, D. and Nattel, S., 2011. Differential protein kinase C isoform regulation and increased constitutive activity of acetylcholine-regulated potassium channels in atrial remodeling. *Circulation research*, 109(9), pp.1031-1043.
252. Maltsev, V.A. and Lakatta, E.G., 2007. Normal heart rhythm is initiated and regulated by an intracellular calcium clock within pacemaker cells. *Heart, Lung and Circulation*, 16(5), pp.335-348.
253. Maltsev, V.A., Rohwedel, J., Hescheler, J. and Wobus, A.M., 1993. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mechanisms of development*, 44(1), pp.41-50.

254. Mannhardt, I., Saleem, U., Mosqueira, D., Loos, M.F., Ulmer, B.M., Lemoine, M.D., Larsson, C., Améen, C., de Korte, T., Vlaming, M.L. and Harris, K., 2020. Comparison of 10 control hPSC lines for drug screening in an engineered heart tissue format. *Stem cell reports*, 15(4), pp.983-998.
255. Markides, V. and Schilling, R.J., 2003. Atrial fibrillation: classification, pathophysiology, mechanisms and drug treatment. *Heart*, 89(8), p.939.
256. Martin, T.G. and Kirk, J.A., 2020. Under construction: the dynamic assembly, maintenance, and degradation of the cardiac sarcomere. *Journal of molecular and cellular cardiology*, 148, pp.89-102.
257. Martino, T.A., Liu, P. and Sole, M.J., 1994. Viral infection and the pathogenesis of dilated cardiomyopathy. *Circulation research*, 74(2), pp.182-188.
258. Mayans, O., van der Ven, P.F., Wilm, M., Mues, A., Young, P., Fürst, D.O., Wilmanns, M. and Gautel, M., 1998. Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature*, 395(6705), pp.863-869.
259. McAfee, Q., Caporizzo, M.A., Uchida, K., Bedi Jr, K.C., Margulies, K.B., Arany, Z. and Prosser, B.L., 2023. Truncated titin protein in dilated cardiomyopathy incorporates into the sarcomere and transmits force. *The Journal of Clinical Investigation*.
260. McAfee, Q., Chen, C.Y., Yang, Y., Caporizzo, M.A., Morley, M., Babu, A., Jeong, S., Brandimarto, J., Bedi Jr, K.C., Flam, E. and Cesare, J., 2021. Truncated titin proteins in dilated cardiomyopathy. *Science Translational Medicine*, 13(618), p.eabd7287.
261. McDonald, K.S., Hanft, L.M., Robinett, J.C., Guglin, M. and Campbell, K.S., 2020. Regulation of myofilament contractile function in human donor and failing hearts. *Frontiers in Physiology*, 11, p.468.
262. McNair, W.P., Sinagra, G., Taylor, M.R., Di Lenarda, A., Ferguson, D.A., Salcedo, E.E., Slavov, D., Zhu, X., Caldwell, J.H., Mestroni, L. and Familial Cardiomyopathy Registry Research Group, 2011. SCN5A mutations associate with arrhythmic dilated cardiomyopathy and commonly localize to the voltage-sensing mechanism. *Journal of the American College of Cardiology*, 57(21), pp.2160-2168.
263. McNally, E.M., Golbus, J.R. and Puckelwartz, M.J., 2013. Genetic mutations and mechanisms in dilated cardiomyopathy. *The Journal of clinical investigation*, 123(1), pp.19-26.
264. Meinertz, T., Hofmann, T., Kasper, W., Treese, N., Bechtold, H., Stenen, U., Pop, T., Leitner, E.R.V., Andresen, D. and Meyer, J., 1984. Significance of ventricular

- arrhythmias in idiopathic dilated cardiomyopathy. *The American journal of cardiology*, 53(7), pp.902-907.
265. Merlo, M., Pivetta, A., Pinamonti, B., Stolfo, D., Zecchin, M., Barbati, G., Di Lenarda, A. and Sinagra, G., 2014. Long-term prognostic impact of therapeutic strategies in patients with idiopathic dilated cardiomyopathy: changing mortality over the last 30 years. *European journal of heart failure*, 16(3), pp.317-324.
 266. 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. and Plowright, A.T., 2017. Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte cell cycle arrest. *Proceedings of the National Academy of Sciences*, 114(40), pp.E8372-E8381.
 267. Miragoli, M., Gaudesius, G. and Rohr, S., 2006. Electrotonic modulation of cardiac impulse conduction by myofibroblasts. *Circulation research*, 98(6), pp.801-810.
 268. Moe, G.K. and Abildskov, J.A., 1959. Atrial fibrillation as a self-sustaining arrhythmia independent of focal discharge. *American heart journal*, 58(1), pp.59-70.
 269. Mohr, E., Thum, T. and Bär, C., 2022. Accelerating cardiovascular research: Recent advances in translational 2D and 3D heart models. *European Journal of Heart Failure*, 24(10), pp.1778-1791.
 270. Moita, M.R., Silva, M.M., Diniz, C., Serra, M., Hoet, R.M., Barbas, A. and Simão, D., 2022. Transcriptome and proteome profiling of activated cardiac fibroblasts supports target prioritization in cardiac fibrosis. *Frontiers in cardiovascular medicine*, 9, p.1015473.
 271. Molkentin, J.D., Bugg, D., Ghearing, N., Dorn, L.E., Kim, P., Sargent, M.A., Gunaje, J., Otsu, K. and Davis, J., 2017. Fibroblast-specific genetic manipulation of p38 mitogen-activated protein kinase in vivo reveals its central regulatory role in fibrosis. *Circulation*, 136(6), pp.549-561.
 272. Morand, J., Arnaud, C., Pepin, J.L. and Godin-Ribuot, D., 2018. Chronic intermittent hypoxia promotes myocardial ischemia-related ventricular arrhythmias and sudden cardiac death. *Scientific reports*, 8(1), pp.1-8.
 273. Morris, T.A., Naik, J., Fibben, K.S., Kong, X., Kiyono, T., Yokomori, K. and Grosberg, A., 2020. Striated myocyte structural integrity: Automated analysis of sarcomeric z-discs. *PLoS computational biology*, 16(3), p.e1007676.
 274. Mosqueira, D., Mannhardt, I., Bhagwan, J.R., Lis-Slimak, K., Katili, P., Scott, E., Hassan, M., Prondzynski, M., Harmer, S.C., Tinker, A. and Smith, J.G., 2018.

- CRISPR/Cas9 editing in human pluripotent stem cell-cardiomyocytes highlights arrhythmias, hypocontractility, and energy depletion as potential therapeutic targets for hypertrophic cardiomyopathy. *European heart journal*, 39(43), pp.3879-3892.
275. Mozaffarian, D., Wilson, P.W. and Kannel, W.B., 2008. Beyond established and novel risk factors: lifestyle risk factors for cardiovascular disease. *Circulation*, 117(23), pp.3031-3038.
 276. Mues, A., van der Ven, P.F., Young, P., Fürst, D.O. and Gautel, M., 1998. Two immunoglobulin-like domains of the Z-disc portion of titin interact in a conformation-dependent way with telethonin. *Febs Letters*, 428(1-2), pp.111-114.
 277. Muhle-Goll, C., Habeck, M., Cazorla, O., Nilges, M., Labeit, S. and Granzier, H., 2001. Structural and functional studies of titin's fn3 modules reveal conserved surface patterns and binding to myosin S1-a possible role in the frank-starling mechanism of the heart. *Journal of molecular biology*, 313(2), pp.431-447.
 278. Musa, H., Meek, S., Gautel, M., Peddie, D., Smith, A.J. and Peckham, M., 2006. Targeted homozygous deletion of M-band titin in cardiomyocytes prevents sarcomere formation. *Journal of cell science*, 119(20), pp.4322-4331.
 279. Muszkiewicz, A., Britton, O.J., Gemmell, P., Passini, E., Sánchez, C., Zhou, X., Carusi, A., Quinn, T.A., Burrage, K., Bueno-Orovio, A. and Rodriguez, B., 2016. Variability in cardiac electrophysiology: using experimentally-calibrated populations of models to move beyond the single virtual physiological human paradigm. *Progress in biophysics and molecular biology*, 120(1-3), pp.115-127.
 280. Nagaraju, C.K., Robinson, E.L., Abdesselem, M., Trenson, S., Dries, E., Gilbert, G., Janssens, S., Van Cleemput, J., Rega, F., Meyns, B. and Roderick, H.L., 2019. Myofibroblast phenotype and reversibility of fibrosis in patients with end-stage heart failure. *Journal of the American College of Cardiology*, 73(18), pp.2267-2282.
 281. Nagueh, S.F., Shah, G., Wu, Y., Torre-Amione, G., King, N.M., Lahmers, S., Witt, C.C., Becker, K., Labeit, S. and Granzier, H.L., 2004. Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. *Circulation*, 110(2), pp.155-162.
 282. Naito, H., Melnychenko, I., Didié, M., Schneiderbanger, K., Schubert, P., Rosenkranz, S., Eschenhagen, T. and Zimmermann, W.H., 2006. Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. *Circulation*, 114(1_supplement), pp.I-72.

283. Nalliah, C.J., Sanders, P., Kottkamp, H. and Kalman, J.M., 2016. The role of obesity in atrial fibrillation. *European heart journal*, 37(20), pp.1565-1572.
284. Nattel, S. and Harada, M., 2014. Atrial remodeling and atrial fibrillation: recent advances and translational perspectives. *Journal of the American College of Cardiology*, 63(22), pp.2335-2345.
285. Nattel, S., Burstein, B. and Dobrev, D., 2008. Atrial remodeling and atrial fibrillation: mechanisms and implications. *Circulation: Arrhythmia and Electrophysiology*, 1(1), pp.62-73.
286. Nattel, S., Heijman, J., Zhou, L. and Dobrev, D., 2020. Molecular basis of atrial fibrillation pathophysiology and therapy: a translational perspective. *Circulation research*, 127(1), pp.51-72.
287. Navarro-Polanco, R.A., Aréchiga-Figueroa, I.A., Salazar-Fajardo, P.D., Benavides-Haro, D.E., Rodríguez-Elías, J.C., Sachse, F.B., Tristani-Firouzi, M., Sánchez-Chapula, J.A. and Moreno-Galindo, E.G., 2013. Voltage sensitivity of M2 muscarinic receptors underlies the delayed rectifier-like activation of ACh-gated K⁺ current by choline in feline atrial myocytes. *The Journal of Physiology*, 591(17), pp.4273-4286.
288. Neumar, R.W., Eigel, B., Callaway, C.W., Estes III, N.M., Jollis, J.G., Kleinman, M.E., Morrison, L.J., Peberdy, M.A., Rabinstein, A., Rea, T.D. and Sendelbach, S., 2015. American Heart Association response to the 2015 Institute of Medicine report on strategies to improve cardiac arrest survival. *Circulation*, 132(11), pp.1049-1070.
289. Nielsen, J.B., Fritsche, L.G., Zhou, W., Teslovich, T.M., Holmen, O.L., Gustafsson, S., Gabrielsen, M.E., Schmidt, E.M., Beaumont, R., Wofford, B.N. and Lin, M., 2018. Genome-wide study of atrial fibrillation identifies seven risk loci and highlights biological pathways and regulatory elements involved in cardiac development. *The American Journal of Human Genetics*, 102(1), pp.103-115.
290. Nishii, K., Shibata, Y. and Kobayashi, Y., 2014. Connexin mutant embryonic stem cells and human diseases. *World journal of stem cells*, 6(5), p.571.
291. Niwa, N. and Nerbonne, J.M., 2010. Molecular determinants of cardiac transient outward potassium current (I_{to}) expression and regulation. *Journal of molecular and cellular cardiology*, 48(1), pp.12-25.
292. Njoroge, J.N., Mangena, J.C., Aribéana, C. and Parikh, V.N., 2022. Emerging Genotype–Phenotype Associations in Dilated Cardiomyopathy. *Current Cardiology Reports*, 24(9), pp.1077-1084.

293. Noh, H., Yu, M.R., Kim, H.J., Lee, J.H., Park, B.W., Wu, I.H., Matsumoto, M. and King, G.L., 2017. Beta 2-adrenergic receptor agonists are novel regulators of macrophage activation in diabetic renal and cardiovascular complications. *Kidney international*, 92(1), pp.101-113.
294. O'Brien, S., Holmes, A.P., Johnson, D.M., Kabir, S.N., O'Shea, C., O'Reilly, M., Avezzu, A., Reyat, J.S., Hall, A.W., Apicella, C. and Ellinor, P.T., 2022. Increased atrial effectiveness of flecainide conferred by altered biophysical properties of sodium channels. *Journal of Molecular and Cellular Cardiology*, 166, pp.23-35.
295. O'Dwyer, S.C., Palacio, S., Matsumoto, C., Guarina, L., Klug, N.R., Tajada, S., Rosati, B., McKinnon, D., Trimmer, J.S. and Santana, L.F., 2020. Kv2. 1 channels play opposing roles in regulating membrane potential, Ca²⁺ channel function, and myogenic tone in arterial smooth muscle. *Proceedings of the National Academy of Sciences*, 117(7), pp.3858-3866.
296. O'Neill, M.D., Jaïs, P., Hocini, M., Sacher, F., Klein, G.J., Clémenty, J. and Haïssaguerre, M., 2007. Catheter ablation for atrial fibrillation. *Circulation*, 116(13), pp.1515-1523.
297. Ojima, K., Ono, Y., Hata, S., Koyama, S., Doi, N. and Sorimachi, H., 2005. Possible functions of p94 in connectin-mediated signaling pathways in skeletal muscle cells. *Journal of Muscle Research & Cell Motility*, 26(6-8), pp.409-417.
298. Olsson, L.G., Swedberg, K., Ducharme, A., Granger, C.B., Michelson, E.L., McMurray, J.J., Puu, M., Yusuf, S., Pfeffer, M.A. and CHARM Investigators, 2006. Atrial fibrillation and risk of clinical events in chronic heart failure with and without left ventricular systolic dysfunction: results from the Candesartan in Heart failure-Assessment of Reduction in Mortality and morbidity (CHARM) program. *Journal of the American College of Cardiology*, 47(10), pp.1997-2004.
299. Ong, L.P., Bargehr, J., Knight-Schrijver, V.R., Lee, J., Colzani, M., Bayraktar, S., Bernard, W.G., Marchiano, S., Bertero, A., Murry, C.E. and Gambardella, L., 2023. Epicardially secreted fibronectin drives cardiomyocyte maturation in 3D-engineered heart tissues. *Stem Cell Reports*, 18(4), pp.936-951.
300. Ongstad, E. and Kohl, P., 2016. Fibroblast–myocyte coupling in the heart: Potential relevance for therapeutic interventions. *Journal of molecular and cellular cardiology*, 91, pp.238-246.
301. Pandit, S.V. and Jalife, J., 2013. Rotors and the dynamics of cardiac fibrillation. *Circulation research*, 112(5), pp.849-862.

302. Panfilov, A.V. and Pertsov, A.M., 1982. Mechanism of the origin of the helical waves in active media associated with the phenomenon of critical curvature. *Biophysics*, 27(5), pp.931-934.
303. Pappireddi, N., Martin, L. and Wühr, M., 2019. A review on quantitative multiplexed proteomics. *ChemBioChem*, 20(10), pp.1210-1224.
304. Pathak, R.K., Mahajan, R., Lau, D.H. and Sanders, P., 2015. The implications of obesity for cardiac arrhythmia mechanisms and management. *Canadian Journal of Cardiology*, 31(2), pp.203-210.
305. Peled, Y., Gramlich, M., Yoskovitz, G., Feinberg, M.S., Afek, A., Polak-Charcon, S., Pras, E., Sela, B.A., Konen, E., Weissbrod, O. and Geiger, D., 2014. Titin mutation in familial restrictive cardiomyopathy. *International journal of cardiology*, 171(1), pp.24-30.
306. Pelin, K., Ridanpää, M., Donner, K., Wilton, S., Krishnarajah, J., Laing, N., Kolmerer, B., Millevoi, S., Labeit, S., de la Chapelle, A. and Wallgren-Pettersson, C., 1997. Refined localisation of the genes for nebulin and titin on chromosome 2q allows the assignment of nebulin as a candidate gene for autosomal recessive nemaline myopathy. *European Journal of Human Genetics*, 5, pp.229-234.
307. Pellman, J., Lyon, R.C. and Sheikh, F., 2010. Extracellular matrix remodeling in atrial fibrosis: mechanisms and implications in atrial fibrillation. *Journal of molecular and cellular cardiology*, 48(3), pp.461-467.
308. Pellman, J., Zhang, J. and Sheikh, F., 2016. Myocyte-fibroblast communication in cardiac fibrosis and arrhythmias: Mechanisms and model systems. *Journal of molecular and cellular cardiology*, 94, pp.22-31.
309. Peng, J., Raddatz, K., Molkentin, J.D., Wu, Y., Labeit, S., Granzier, H. and Gotthardt, M., 2007. Cardiac hypertrophy and reduced contractility in hearts deficient in the titin kinase region. *Circulation*, 115(6), pp.743-751.
310. Peters, S., Kumar, S., Elliott, P., Kalman, J.M. and Fatkin, D., 2019. Arrhythmic genotypes in familial dilated cardiomyopathy: implications for genetic testing and clinical management. *Heart, Lung and Circulation*, 28(1), pp.31-38.
311. Petrov, V.V., Fagard, R.H. and Lijnen, P.J., 2002. Stimulation of collagen production by transforming growth factor- β 1 during differentiation of cardiac fibroblasts to myofibroblasts. *Hypertension*, 39(2), pp.258-263.

312. Pinto, A.R., Ilinykh, A., Ivey, M.J., Kuwabara, J.T., D'antoni, M.L., Debuque, R., Chandran, A., Wang, L., Arora, K., Rosenthal, N.A. and Tallquist, M.D., 2016. Revisiting cardiac cellular composition. *Circulation research*, 118(3), pp.400-409.
313. Piroddi, N., Belus, A., Scellini, B., Tesi, C., Giunti, G., Cerbai, E., Mugelli, A. and Poggesi, C., 2007. Tension generation and relaxation in single myofibrils from human atrial and ventricular myocardium. *Pflügers Archiv-European Journal of Physiology*, 454(1), pp.63-73.
314. Plant, L.D., Xiong, D., Romero, J., Dai, H. and Goldstein, S.A., 2020. Hypoxia produces pro-arrhythmic late sodium current in cardiac myocytes by SUMOylation of NaV1.5 channels. *Cell reports*, 30(7), pp.2225-2236.
315. Poulet, C., Künzel, S., Büttner, E., Lindner, D., Westermann, D. and Ravens, U., 2016. Altered physiological functions and ion currents in atrial fibroblasts from patients with chronic atrial fibrillation. *Physiological reports*, 4(2), p.e12681.
316. Pozzoli, M., Cioffi, G., Traversi, E., Pinna, G.D., Cobelli, F. and Tavazzi, L., 1998. Predictors of primary atrial fibrillation and concomitant clinical and hemodynamic changes in patients with chronic heart failure: a prospective study in 344 patients with baseline sinus rhythm. *Journal of the American College of Cardiology*, 32(1), pp.197-204.
317. Prystowsky, E.N., Padanilam, B.J. and Fogel, R.I., 2015. Treatment of atrial fibrillation. *Jama*, 314(3), pp.278-288.
318. Qi, X.Y., Huang, H., Ordog, B., Luo, X., Naud, P., Sun, Y., Wu, C.T., Dawson, K., Tadevosyan, A., Chen, Y. and Harada, M., 2015. Fibroblast inward-rectifier potassium current upregulation in profibrillatory atrial remodeling. *Circulation research*, 116(5), pp.836-845.
319. Radke, M.H., Polack, C., Methawasin, M., Fink, C., Granzier, H.L. and Gotthardt, M., 2019. Deleting full length titin versus the titin M-band region leads to differential mechanosignaling and cardiac phenotypes. *Circulation*, 139(15), pp.1813-1827.
320. Raica, M. and Cimpean, A.M., 2010. Platelet-derived growth factor (PDGF)/PDGF receptors (PDGFR) axis as target for antitumor and antiangiogenic therapy. *pharmaceuticals*, 3(3), pp.572-599.
321. Raines, E.W., 2004. PDGF and cardiovascular disease. *Cytokine & growth factor reviews*, 15(4), pp.237-254.
322. Ramos, F.J., Chen, S.C., Garelick, M.G., Dai, D.F., Liao, C.Y., Schreiber, K.H., MacKay, V.L., An, E.H., Strong, R., Ladiges, W.C. and Rabinovitch, P.S., 2012.

- Rapamycin reverses elevated mTORC1 signaling in lamin A/C-deficient mice, rescues cardiac and skeletal muscle function, and extends survival. *Science translational medicine*, 4(144), pp.144ra103-144ra103.
323. Ravenscroft, S.M., Pointon, A., Williams, A.W., Cross, M.J. and Sidaway, J.E., 2016. Cardiac non-myocyte cells show enhanced pharmacological function suggestive of contractile maturity in stem cell derived cardiomyocyte microtissues. *Toxicological Sciences*, 152(1), pp.99-112.
 324. Reyat, J.S., Chua, W., Cardoso, V.R., Witten, A., Kastner, P.M., Kabir, S.N., Sinner, M.F., Wesselink, R., Holmes, A.P., Pavlovic, D. and Stoll, M., 2020. Reduced left atrial cardiomyocyte PITX2 and elevated circulating BMP10 predict atrial fibrillation after ablation. *JCI insight*, 5(16).
 325. Rivera-Arbeláez, J.M., Cofiño-Fabres, C., Schwach, V., Boonen, T., Ten Den, S.A., Vermeul, K., van den Berg, A., Segerink, L.I., Ribeiro, M.C. and Passier, R., 2022. Contractility analysis of human engineered 3D heart tissues by an automatic tracking technique using a standalone application. *Plos one*, 17(4), p.e0266834.
 326. Rizvi, F., DeFranco, A., Siddiqui, R., Negmadjanov, U., Emelyanova, L., Holmuhamedov, A., Ross, G., Shi, Y., Holmuhamedov, E., Kress, D. and Tajik, A.J., 2016. Chamber-specific differences in human cardiac fibroblast proliferation and responsiveness toward simvastatin. *American Journal of Physiology-Cell Physiology*, 311(2), pp.C330-C339.
 327. Roberts, A.M., Ware, J.S., Herman, D.S., Schafer, S., Baksi, J., Bick, A.G., Buchan, R.J., Walsh, R., John, S., Wilkinson, S. and Mazzarotto, F., 2015. Integrated allelic, transcriptional, and phenomic dissection of the cardiac effects of titin truncations in health and disease. *Science translational medicine*, 7(270), pp.270ra6-270ra6.
 328. Rodríguez-Pascual, F., Busnadiego, O., Lagares, D. and Lamas, S., 2011. Role of endothelin in the cardiovascular system. *Pharmacological research*, 63(6), pp.463-472.
 329. Rohr, S., 2004. Role of gap junctions in the propagation of the cardiac action potential. *Cardiovascular research*, 62(2), pp.309-322.
 330. Ronaldson-Bouchard, K., Ma, S.P., Yeager, K., Chen, T., Song, L., Sirabella, D., Morikawa, K., Teles, D., Yazawa, M. and Vunjak-Novakovic, G., 2018. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature*, 556(7700), pp.239-243.
 331. Roselli, C., Chaffin, M.D., Weng, L.C., Aeschbacher, S., Ahlberg, G., Albert, C.M., Almgren, P., Alonso, A., Anderson, C.D., Aragam, K.G. and Arking, D.E., 2018. Multi-

- ethnic genome-wide association study for atrial fibrillation. *Nature genetics*, 50(9), pp.1225-1233.
332. Roselli, C., Rienstra, M. and Ellinor, P.T., 2020. Genetics of atrial fibrillation in 2020: GWAS, genome sequencing, polygenic risk, and beyond. *Circulation research*, 127(1), pp.21-33.
 333. Sadoshima, J.I., Xu, Y., Slayter, H.S. and Izumo, S., 1993. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell*, 75(5), pp.977-984.
 334. Saleeb-Mousa, J., Nathanael, D., Coney, A.M., Kalla, M., Brain, K.L. and Holmes, A.P., 2023. Mechanisms of atrial fibrillation in obstructive sleep apnoea. *Cells*, 12(12), p.1661.
 335. Salem, T., Frankman, Z. and Churko, J.M., 2021. Tissue Engineering Techniques for Induced Pluripotent Stem Cell Derived Three-Dimensional Cardiac Constructs. *Tissue Engineering Part B: Reviews*.
 336. Sánchez, C., Bueno-Orovio, A., Wettwer, E., Loose, S., Simon, J., Ravens, U., Pueyo, E. and Rodriguez, B., 2014. Inter-subject variability in human atrial action potential in sinus rhythm versus chronic atrial fibrillation. *PloS one*, 9(8), p.e105897.
 337. Sano, M., Fukuda, K., Sato, T., Kawaguchi, H., Suematsu, M., Matsuda, S., Koyasu, S., Matsui, H., Yamauchi-Takahara, K., Harada, M. and Saito, Y., 2001. ERK and p38 MAPK, but not NF- κ B, are critically involved in reactive oxygen species-mediated induction of IL-6 by angiotensin II in cardiac fibroblasts. *Circulation research*, 89(8), pp.661-669.
 338. Sarparanta, J., Blandin, G., Charton, K., Vihola, A., Marchand, S., Milic, A., Hackman, P., Ehler, E., Richard, I. and Udd, B., 2010. Interactions with M-band titin and calpain 3 link myospryn (CMYA5) to tibial and limb-girdle muscular dystrophies. *Journal of Biological Chemistry*, 285(39), pp.30304-30315.
 339. Satoh, M., Takahashi, M., Sakamoto, T., Hiroe, M., Marumo, F. and Kimura, A., 1999. Structural analysis of the titin gene in hypertrophic cardiomyopathy: identification of a novel disease gene. *Biochemical and biophysical research communications*, 262(2), pp.411-417.
 340. Savarese, M., Sarparanta, J., Vihola, A., Udd, B. and Hackman, P., 2016. Increasing role of titin mutations in neuromuscular disorders. *Journal of neuromuscular diseases*, 3(3), pp.293-308.

341. Savla, J.J., Nelson, B.C., Perry, C.N. and Adler, E.D., 2014. Induced pluripotent stem cells for the study of cardiovascular disease. *Journal of the American College of Cardiology*, 64(5), pp.512-519.
342. Schaaf, S., Shibamiya, A., Mewe, M., Eder, A., Stöhr, A., Hirt, M.N., Rau, T., Zimmermann, W.H., Conradi, L., Eschenhagen, T. and Hansen, A., 2011. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. *PloS one*, 6(10), p.e26397.
343. Schafer, S., De Marvao, A., Adami, E., Fiedler, L.R., Ng, B., Khin, E., Rackham, O.J., Van Heesch, S., Pua, C.J., Kui, M. and Walsh, R., 2017. Titin-truncating variants affect heart function in disease cohorts and the general population. *Nature genetics*, 49(1), pp.46-53.
344. Schick, R., Mekies, L.N., Shemer, Y., Eisen, B., Hallas, T., Ben Jehuda, R., Ben-Ari, M., Szantai, A., Willi, L., Shulman, R. and Gramlich, M., 2018. Functional abnormalities in induced pluripotent stem cell-derived cardiomyocytes generated from titin-mutated patients with dilated cardiomyopathy. *PLoS One*, 13(10), p.e0205719.
345. Schlittler, M., Pramstaller, P.P., Rossini, A. and De Bortoli, M., 2023. Myocardial Fibrosis in Hypertrophic Cardiomyopathy: A Perspective from Fibroblasts. *International Journal of Molecular Sciences*, 24(19), p.14845.
346. Schmittgen, T.D. and Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols*, 3(6), pp.1101-1108.
347. Schoonderwoerd, B.A., Van Gelder, I.C., Van Veldhuisen, D.J., Van den Berg, M.P. and Crijns, H.J., 2005. Electrical and structural remodeling: role in the genesis and maintenance of atrial fibrillation. *Progress in cardiovascular diseases*, 48(3), pp.153-168.
348. Schultheiss, H.P., Fairweather, D., Caforio, A.L., Escher, F., Hershberger, R.E., Lipshultz, S.E., Liu, P.P., Matsumori, A., Mazzanti, A., McMurray, J. and Priori, S.G., 2019. Dilated cardiomyopathy. *Nature reviews Disease primers*, 5(1), p.32.
349. Schultz, F., Swiatlowska, P., Alvarez-Laviada, A., Sanchez-Alonso, J.L., Song, Q., de Vries, A.A., Pijnappels, D.A., Ongstad, E., Braga, V.M., Entcheva, E. and Gourdie, R.G., 2019. Cardiomyocyte–myofibroblast contact dynamism is modulated by connexin-43. *The FASEB Journal*, 33(9), p.10453.
350. Schulz, C., Lemoine, M.D., Mearini, G., Koivumäki, J., Sani, J., Schwedhelm, E., Kirchhof, P., Ghalawinji, A., Stoll, M., Hansen, A. and Eschenhagen, T., 2023. PITX2 Knockout Induces Key Findings of Electrical Remodeling as Seen in Persistent Atrial Fibrillation. *Circulation: Arrhythmia and Electrophysiology*, p.e011602.

351. Schweizer, P.A., Darche, F.F., Ullrich, N.D., Geschwill, P., Greber, B., Rivinius, R., Seyler, C., Müller-Decker, K., Draguhn, A., Utikal, J. and Koenen, M., 2017. Subtype-specific differentiation of cardiac pacemaker cell clusters from human induced pluripotent stem cells. *Stem cell research & therapy*, 8(1), pp.1-15.
352. Segura, A.M., Frazier, O.H. and Buja, L.M., 2014. Fibrosis and heart failure. *Heart failure reviews*, 19, pp.173-185.
353. Seibertz, F., Sutanto, H., Dülk, R., Pronto, J.R.D., Springer, R., Rapedius, M., Liutkute, A., Ritter, M., Jung, P., Stelzer, L. and Hüsken, L.M., 2023. Electrophysiological and calcium-handling development during long-term culture of human-induced pluripotent stem cell-derived cardiomyocytes. *Basic Research in Cardiology*, 118(1), p.14.
354. Shadrin, I.Y., Allen, B.W., Qian, Y., Jackman, C.P., Carlson, A.L., Juhas, M.E. and Bursac, N., 2017. Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nature communications*, 8(1), pp.1-15.
355. Sheng, J., Shim, W., Wei, H., Lim, S.Y., Liew, R., Lim, T.S., Ong, B.H., Chua, Y.L. and Wong, P., 2013. Hydrogen sulphide suppresses human atrial fibroblast proliferation and transformation to myofibroblasts. *Journal of Cellular and Molecular Medicine*, 17(10), pp.1345-1354.
356. Shi-Wen, X., Chen, Y., Denton, C.P., Eastwood, M., Renzoni, E.A., Bou-Gharios, G., Pearson, J.D., Dashwood, M., du Bois, R.M., Black, C.M. and Leask, A., 2004. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Molecular biology of the cell*, 15(6), pp.2707-2719.
357. Shi-wen, X., Kennedy, L., Renzoni, E.A., Bou-Gharios, G., du Bois, R.M., Black, C.M., Denton, C.P., Abraham, D.J. and Leask, A., 2007. Endothelin is a downstream mediator of profibrotic responses to transforming growth factor β in human lung fibroblasts. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology*, 56(12), pp.4189-4194.
358. Silbernagel, N., Körner, A., Balitzki, J., Jaggy, M., Bertels, S., Richter, B., Hippler, M., Hellwig, A., Hecker, M., Bastmeyer, M. and Ullrich, N.D., 2020. Shaping the heart: Structural and functional maturation of iPSC-cardiomyocytes in 3D-micro-scaffolds. *Biomaterials*, 227, p.119551.

359. Simm, A., Nestler, M. and Hoppe, V., 1998. Mitogenic effect of PDGF-AA on cardiac fibroblasts. *Basic research in cardiology*, 93(Suppl 3), pp.s040-s043.
360. Siu, B.L., Niimura, H., Osborne, J.A., Fatkin, D., MacRae, C., Solomon, S., Benson, D.W., Seidman, J.G. and Seidman, C.E., 1999. Familial dilated cardiomyopathy locus maps to chromosome 2q31. *Circulation*, 99(8), pp.1022-1026.
361. Skorska, A., Johann, L., Chabanovska, O., Vasudevan, P., Kussauer, S., Hillemanns, M., Wolfien, M., Jonitz-Heincke, A., Wolkenhauer, O., Bader, R. and Lang, H., 2022. Monitoring the maturation of the sarcomere network: A super-resolution microscopy-based approach. *Cellular and Molecular Life Sciences*, 79(3), pp.1-16.
362. Smith, A.S., Macadangdang, J., Leung, W., Laflamme, M.A. and Kim, D.H., 2017. Human iPSC-derived cardiomyocytes and tissue engineering strategies for disease modeling and drug screening. *Biotechnology advances*, 35(1), pp.77-94.
363. Smith, J.G., Owen, T., Bhagwan, J.R., Mosqueira, D., Scott, E., Mannhardt, I., Patel, A., Barriales-Villa, R., Monserrat, L., Hansen, A. and Eschenhagen, T., 2018. Isogenic pairs of iPSC-CMs with hypertrophic cardiomyopathy/LVNC-associated ACTC1 E99K mutation unveil differential functional deficits. *Stem cell reports*, 11(5), pp.1226-1243.
364. Snider, P., Standley, K.N., Wang, J., Azhar, M., Doetschman, T. and Conway, S.J., 2009. Origin of cardiac fibroblasts and the role of periostin. *Circulation research*, 105(10), pp.934-947.
365. Snyders, D.J., 1999. Structure and function of cardiac potassium channels. *Cardiovascular research*, 42(2), pp.377-390.
366. Sohns, C. and Marrouche, N.F., 2020. Atrial fibrillation and cardiac fibrosis. *European heart journal*, 41(10), pp.1123-1131.
367. Sottas, V., Wahl, C.M., Trache, M.C., Bartolf-Kopp, M., Cambridge, S., Hecker, M. and Ullrich, N.D., 2018. Improving electrical properties of iPSC-cardiomyocytes by enhancing Cx43 expression. *Journal of Molecular and Cellular Cardiology*, 120, pp.31-41.
368. Souders, C.A., Bowers, S.L. and Baudino, T.A., 2009. Cardiac fibroblast: the renaissance cell. *Circulation research*, 105(12), pp.1164-1176.
369. Soussi, S., Savchenko, L., Rovina, D., Iacovoni, J.S., Gottinger, A., Vialettes, M., Pioner, J.M., Farini, A., Mallia, S., Rabino, M. and Pompilio, G., 2023. iPSC derived cardiac fibroblasts of DMD patients show compromised actin microfilaments, metabolic shift and pro-fibrotic phenotype. *Biology direct*, 18(1), p.41.

370. Spezzacatene, A., Sinagra, G., Merlo, M., Barbati, G., Graw, S.L., Brun, F., Slavov, D., Di Lenarda, A., Salcedo, E.E., Towbin, J.A. and Saffitz, J.E., 2015. Arrhythmogenic phenotype in dilated cardiomyopathy: natural history and predictors of life-threatening arrhythmias. *Journal of the American Heart Association*, 4(10), p.e002149.
371. Spracklen, T.F., Chakafana, G., Schwartz, P.J., Kotta, M.C., Shaboodien, G., Ntusi, N.A. and Sliwa, K., 2021. Genetics of peripartum cardiomyopathy: current knowledge, future directions and clinical implications. *Genes*, 12(1), p.103.
372. Sridhar, S., Vandersickel, N. and Panfilov, A.V., 2017. Effect of myocyte-fibroblast coupling on the onset of pathological dynamics in a model of ventricular tissue. *Scientific reports*, 7(1), p.40985.
373. Stavrakis, S. and Po, S., 2017. Ganglionated plexi ablation: physiology and clinical applications. *Arrhythmia & electrophysiology review*, 6(4), p.186.
374. Stewart, L. and Turner, N.A., 2021. Channelling the force to reprogram the matrix: Mechanosensitive ion channels in cardiac fibroblasts. *Cells*, 10(5), p.990.
375. Stockand, J.D. and Meszaros, J.G., 2003. Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. *American Journal of Physiology-Heart and Circulatory Physiology*, 284(1), pp.H176-H184.
376. Stroeks, S.L., van Beek, D., Adriaens, M.E. and Verdonchot, J.A., 2023. Titin Allelic Expression and Protein Processing Pathways in Early-Stage Dilated Cardiomyopathy Patients With Truncating Titin Variants. *Circulation: Genomic and Precision Medicine*, 16(1), p.e003901.
377. Syeda, F., Holmes, A.P., Yu, T.Y., Tull, S., Kuhlmann, S.M., Pavlovic, D., Betney, D., Riley, G., Kucera, J.P., Jousset, F. and de Groot, J.R., 2016. PITX2 modulates atrial membrane potential and the antiarrhythmic effects of sodium-channel blockers. *Journal of the American College of Cardiology*, 68(17), pp.1881-1894.
378. Tabish, A.M., Azzimato, V., Alexiadis, A., Buyandelger, B. and Knöll, R., 2017. Genetic epidemiology of titin-truncating variants in the etiology of dilated cardiomyopathy. *Biophysical Reviews*, 9(3), pp.207-223.
379. Tadano, K., Miyagawa, S., Takeda, M., Tsukamoto, Y., Kazusa, K., Takamatsu, K., Akashi, M. and Sawa, Y., 2021. Cardiotoxicity assessment using 3D vascularized cardiac tissue consisting of human iPSC-derived cardiomyocytes and fibroblasts. *Molecular Therapy-Methods & Clinical Development*, 22, pp.338-349.

380. Takahashi, K. and Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *cell*, 126(4), pp.663-676.
381. Tarbit, E., Singh, I., Peart, J.N. and Rose'Meyer, R.B., 2019. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. *Heart failure reviews*, 24, pp.1-15.
382. Tayal, U., Newsome, S., Buchan, R., Whiffin, N., Walsh, R., Barton, P.J., Ware, J.S., Cook, S.A. and Prasad, S.K., 2017. Truncating variants in titin independently predict early arrhythmias in patients with dilated cardiomyopathy. *Journal of the American College of Cardiology*, 69(19), p.2466.
383. Taylor, M., Graw, S., Sinagra, G., Barnes, C., Slavov, D., Brun, F., Pinamonti, B., Salcedo, E.E., Sauer, W., Pyxaras, S. and Anderson, B., 2011. Genetic variation in titin in arrhythmogenic right ventricular cardiomyopathy—overlap syndromes. *Circulation*, 124(8), pp.876-885.
384. Tchou, P.J., Kadri, N., Anderson, J., Caceres, J.A., Jazayeri, M. and Akhtar, M., 1988. Automatic implantable cardioverter defibrillators and survival of patients with left ventricular dysfunction and malignant ventricular arrhythmias. *Annals of Internal Medicine*, 109(7), pp.529-534.
385. Thavandiran, N., Dubois, N., Mikryukov, A., Massé, S., Beca, B., Simmons, C.A., Deshpande, V.S., McGarry, J.P., Chen, C.S., Nanthakumar, K. and Keller, G.M., 2013. Design and formulation of functional pluripotent stem cell-derived cardiac microtissues. *Proceedings of the National Academy of Sciences*, 110(49), pp.E4698-E4707.
386. Thomas, D., Cunningham, N.J., Shenoy, S. and Wu, J.C., 2022. Human-induced pluripotent stem cells in cardiovascular research: current approaches in cardiac differentiation, maturation strategies, and scalable production. *Cardiovascular research*, 118(1), pp.20-36.
387. Tiburcy, M., Hudson, J.E., Balfanz, P., Schlick, S., Meyer, T., Chang Liao, M.L., Levent, E., Raad, F., Zeidler, S., Wingender, E. and Riegler, J., 2017. Defined engineered human myocardium with advanced maturation for applications in heart failure modeling and repair. *Circulation*, 135(19), pp.1832-1847.
388. Toepfer, C.N., Sharma, A., Cicconet, M., Garfinkel, A.C., Mücke, M., Neyazi, M., Willcox, J.A., Agarwal, R., Schmid, M., Rao, J. and Ewoldt, J., 2019. SarcTrack: an

- adaptable software tool for efficient large-scale analysis of sarcomere function in iPSC-cardiomyocytes. *Circulation research*, 124(8), pp.1172-1183.
389. Trautwein, W., Kassebaum, D.G., Nelson, R.M. and Hecht, H.H., 1962. Electrophysiological study of human heart muscle. *Circulation research*, 10(3), pp.306-312.
390. Travers, J.G., Kamal, F.A., Robbins, J., Yutzey, K.E. and Blaxall, B.C., 2016. Cardiac fibrosis: the fibroblast awakens. *Circulation research*, 118(6), pp.1021-1040.
391. Travers, J.G., Kamal, F.A., Valiente-Alandi, I., Nieman, M.L., Sargent, M.A., Lorenz, J.N., Molkentin, J.D. and Blaxall, B.C., 2017. Pharmacological and activated fibroblast targeting of G β γ -GRK2 after myocardial ischemia attenuates heart failure progression. *Journal of the American College of Cardiology*, 70(8), pp.958-971.
392. Tsao, C.W., Aday, A.W., Almarzooq, Z.I., Alonso, A., Beaton, A.Z., Bittencourt, M.S., Boehme, A.K., Buxton, A.E., Carson, A.P., Commodore-Mensah, Y. and Elkind, M.S., 2022. Heart disease and stroke statistics—2022 update: a report from the American Heart Association. *Circulation*, 145(8), pp.e153-e639.
393. Tskhovrebova, L. and Trinick, J., 2003. Titin: properties and family relationships. *Nature reviews Molecular cell biology*, 4(9), pp.679-689.
394. Tucker, N.R., Chaffin, M., Fleming, S.J., Hall, A.W., Parsons, V.A., Bedi Jr, K.C., Akkad, A.D., Herndon, C.N., Arduini, A., Papangelis, I. and Roselli, C., 2020. Transcriptional and cellular diversity of the human heart. *Circulation*, 142(5), pp.466-482.
395. Tulloch, N.L., Muskheli, V., Razumova, M.V., Korte, F.S., Regnier, M., Hauch, K.D., Pabon, L., Reinecke, H. and Murry, C.E., 2011. Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circulation research*, 109(1), pp.47-59.
396. Tzatzalos, E., Abilez, O.J., Shukla, P. and Wu, J.C., 2016. Engineered heart tissues and induced pluripotent stem cells: macro-and microstructures for disease modeling, drug screening, and translational studies. *Advanced drug delivery reviews*, 96, pp.234-244.
397. Ulmer, B.M., Stoehr, A., Schulze, M.L., Patel, S., Gucek, M., Mannhardt, I., Funcke, S., Murphy, E., Eschenhagen, T. and Hansen, A., 2018. Contractile work contributes to maturation of energy metabolism in iPSC-derived cardiomyocytes. *Stem cell reports*, 10(3), pp.834-847.
398. Umana, E., Solares, C.A. and Alpert, M.A., 2003. Tachycardia-induced cardiomyopathy. *The American journal of medicine*, 114(1), pp.51-55.

399. Vad, O.B., Paludan-Müller, C., Ahlberg, G., Kalstø, S.M., Ghouse, J., Andreassen, L., Haunsø, S., Tveit, A., Sajadieh, A., Christophersen, I.E. and Svendsen, J.H., 2020. Loss-of-function variants in cytoskeletal genes are associated with early-onset atrial fibrillation. *Journal of clinical medicine*, 9(2), p.372.
400. Van Berlo, J.H., De Voogt, W.G., Van Der Kooi, A.J., Van Tintelen, J.P., Bonne, G., Yaou, R.B., Duboc, D., Rossenbacker, T., Heidbüchel, H., De Visser, M. and Crijns, H.J., 2005. Meta-analysis of clinical characteristics of 299 carriers of LMNA gene mutations: do lamin A/C mutations portend a high risk of sudden death?. *Journal of molecular medicine*, 83(1), pp.79-83.
401. Van den Eynde, C., Vriens, J. and De Clercq, K., 2021. Transient receptor potential channel regulation by growth factors. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1868(4), p.118950.
402. Van Der Ven, P.F. and Fürst, D.O., 1997. Assembly of titin, myomesin and M-protein into the sarcomeric M band in differentiating human skeletal muscle cells in vitro. *Cell structure and function*, 22(1), pp.163-171.
403. Van der Ven, P.F., Bartsch, J.W., Gautel, M., Jockusch, H. and Furst, D.O., 2000. A functional knock-out of titin results in defective myofibril assembly. *Journal of Cell Science*, 113(8), pp.1405-1414.
404. van Mil, A., Balk, G.M., Neef, K., Buikema, J.W., Asselbergs, F.W., Wu, S.M., Doevendans, P.A. and Sluijter, J.P., 2018. Modelling inherited cardiac disease using human induced pluripotent stem cell-derived cardiomyocytes: progress, pitfalls, and potential. *Cardiovascular research*, 114(14), pp.1828-1842.
405. van Spaendonck-Zwarts, K.Y., Posafalvi, A., van den Berg, M.P., Hilfiker-Kleiner, D., Bollen, I.A., Sliwa, K., Alders, M., Almomani, R., van Langen, I.M., van der Meer, P. and Sinke, R.J., 2014. Titin gene mutations are common in families with both peripartum cardiomyopathy and dilated cardiomyopathy. *European heart journal*, 35(32), pp.2165-2173.
406. Vasquez, C., Benamer, N. and Morley, G.E., 2011. The cardiac fibroblast: functional and electrophysiological considerations in healthy and diseased hearts. *Journal of cardiovascular pharmacology*, 57(4), p.380.
407. Veerman, C.C., Mengarelli, I., Lodder, E.M., Kosmidis, G., Bellin, M., Zhang, M., Dittmann, S., Guan, K., Wilde, A.A., Schulze-Bahr, E. and Greber, B., 2017. Switch From Fetal to Adult SCN 5A 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), p.e005135.
408. Verdonschot, J.A., Hazebroek, M.R., Derks, K.W., Barandiarán Aizpurua, A., Merken, J.J., Wang, P., Bierau, J., van den Wijngaard, A., Schalla, S.M., Abdul Hamid, M.A. and van Bilsen, M., 2018. Titin cardiomyopathy leads to altered mitochondrial energetics, increased fibrosis and long-term life-threatening arrhythmias. *European heart journal*, 39(10), pp.864-873.
 409. Vikhorev, P.G., Smoktunowicz, N., Munster, A.B., Kostin, S., Montgiraud, C., Messer, A.E., Toliat, M.R., Li, A., Dos Remedios, C.G., Lal, S. and Blair, C.A., 2017. Abnormal contractility in human heart myofibrils from patients with dilated cardiomyopathy due to mutations in TTN and contractile protein genes. *Scientific reports*, 7(1), pp.1-11.
 410. Vikhorev, P.G., Vikhoreva, N.N., Yeung, W., Li, A., Lal, S., Dos Remedios, C.G., Blair, C.A., Guglin, M., Campbell, K.S., Yacoub, M.H. and de Tombe, P., 2020. Titin-truncating mutations associated with dilated cardiomyopathy alter length-dependent activation and its modulation via phosphorylation. *Cardiovascular Research*.
 411. Virani, S.S., Alonso, A., Benjamin, E.J., Bittencourt, M.S., Callaway, C.W., Carson, A.P., Chamberlain, A.M., Chang, A.R., Cheng, S., Delling, F.N. and Djousse, L., 2020. Heart disease and stroke statistics—2020 update: a report from the American Heart Association. *Circulation*, 141(9), pp.e139-e596.
 412. Virchow, R., 1867. *Handbuch der speciellen Pathologie und Therapie* (Vol. 5). Enke.
 413. Vissing, C.R., Rasmussen, T.B., Dybro, A.M., Olesen, M.S., Pedersen, L.N., Jensen, M., Bundgaard, H. and Christensen, A.H., 2021. Dilated cardiomyopathy caused by truncating titin variants: long-term outcomes, arrhythmias, response to treatment and sex differences. *Journal of Medical Genetics*, 58(12), pp.832-841.
 414. Vissing, C.R., Rasmussen, T.B., Olesen, M.S., Pedersen, L.N., Dybro, A., Bundgaard, H., Jensen, M. and Christensen, A.H., 2019. P337 Natural history, reversibility and arrhythmias associated with truncating titin variants in dilated cardiomyopathy. *European Heart Journal*, 40(Supplement_1), pp.eh747-0171.
 415. Vizzardi, E., Curnis, A., Latini, M.G., Salghetti, F., Rocco, E., Lupi, L., Rovetta, R., Quinzani, F., Bonadei, I., Bontempi, L. and D'Aloia, A., 2014. Risk factors for atrial fibrillation recurrence: a literature review. *Journal of cardiovascular medicine*, 15(3), pp.235-253.

416. Voigt, N., Heijman, J., Trausch, A., Mintert-Jancke, E., Pott, L., Ravens, U. and Dobrev, D., 2013. Impaired Na⁺-dependent regulation of acetylcholine-activated inward-rectifier K⁺ current modulates action potential rate dependence in patients with chronic atrial fibrillation. *Journal of molecular and cellular cardiology*, 61, pp.142-152.
417. Vornanen, M. and Hassinen, M., 2016. Zebrafish heart as a model for human cardiac electrophysiology. *Channels*, 10(2), pp.101-110.
418. Vučković, S., Dinani, R., Nollet, E.E., Kuster, D.W., Buikema, J.W., Houtkooper, R.H., Nabben, M., van der Velden, J. and Goversen, B., 2022. Characterization of cardiac metabolism in iPSC-derived cardiomyocytes: lessons from maturation and disease modeling. *Stem Cell Research & Therapy*, 13(1), pp.1-19.
419. Waks, J.W. and Josephson, M.E., 2014. Mechanisms of atrial fibrillation–reentry, rotors and reality. *Arrhythmia & electrophysiology review*, 3(2), p.90.
420. Waldmann, V., Jouven, X., Narayanan, K., Piot, O., Chugh, S.S., Albert, C.M. and Marijon, E., 2020. Association between atrial fibrillation and sudden cardiac death: pathophysiological and epidemiological insights. *Circulation Research*, 127(2), pp.301-309.
421. Wang, G., McCain, M.L., Yang, L., He, A., Pasqualini, F.S., Agarwal, A., Yuan, H., Jiang, D., Zhang, D., Zangi, L. and Geva, J., 2014. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nature medicine*, 20(6), pp.616-623.
422. Wang, L., Yue, Y., Yang, X., Fan, T., Mei, B., Hou, J., Liang, M., Chen, G. and Wu, Z., 2017. Platelet derived growth factor alpha (PDGFR α) induces the activation of cardiac fibroblasts by activating c-kit. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, 23, p.3808.
423. Wang, L.I., Yu, P., Zhou, B., Song, J., Li, Z., Zhang, M., Guo, G., Wang, Y., Chen, X., Han, L. and Hu, S., 2020. Single-cell reconstruction of the adult human heart during heart failure and recovery reveals the cellular landscape underlying cardiac function. *Nature cell biology*, 22(1), pp.108-119.
424. Wang, Y., Xu, H., Kumar, R., Tipparaju, S.M., Wagner, M.B. and Joyner, R.W., 2003. Differences in transient outward current properties between neonatal and adult human atrial myocytes. *Journal of molecular and cellular cardiology*, 35(9), pp.1083-1092.
425. Ware, J.S., Amor-Salamanca, A., Tayal, U., Govind, R., Serrano, I., Salazar-Mendiguchía, J., García-Pinilla, J.M., Pascual-Figal, D.A., Nuñez, J., Guzzo-Merello, G.

- and Gonzalez-Vioque, E., 2018. Genetic etiology for alcohol-induced cardiac toxicity. *Journal of the American College of Cardiology*, 71(20), pp.2293-2302.
426. Ware, J.S., Li, J., Mazaika, E., Yasso, C.M., DeSouza, T., Cappola, T.P., Tsai, E.J., Hilfiker-Kleiner, D., Kamiya, C.A., Mazzarotto, F. and Cook, S.A., 2016. Shared genetic predisposition in peripartum and dilated cardiomyopathies. *New England Journal of Medicine*, 374(3), pp.233-241.
427. Weber, K.T., Sun, Y., Bhattacharya, S.K., Ahokas, R.A. and Gerling, I.C., 2013. Myofibroblast-mediated mechanisms of pathological remodelling of the heart. *Nature Reviews Cardiology*, 10(1), pp.15-26.
428. Werhahn, S.M., Kreusser, J.S., Hagenmüller, M., Beckendorf, J., Diemert, N., Hoffmann, S., Schultz, J.H., Backs, J. and Dewenter, M., 2021. Adaptive versus maladaptive cardiac remodelling in response to sustained β -adrenergic stimulation in a new 'ISO on/off model'. *PLoS One*, 16(6), p.e0248933.
429. Wermuth, P.J., Li, Z., Mendoza, F.A. and Jimenez, S.A., 2016. Stimulation of transforming growth factor- β 1-induced endothelial-to-mesenchymal transition and tissue fibrosis by endothelin-1 (ET-1): a novel profibrotic effect of ET-1. *PLoS One*, 11(9), p.e0161988.
430. Wiesinger, A., Boink, G.J., Christoffels, V.M. and Devalla, H.D., 2021. Retinoic acid signaling in heart development: Application in the differentiation of cardiovascular lineages from human pluripotent stem cells. *Stem Cell Reports*, 16(11), pp.2589-2606.
431. Wiggins, B.S., Cibotti-Sun, M. and Moore, M.M., 2024. 2023 Atrial Fibrillation Guideline-at-a-Glance. *Journal of the American College of Cardiology*, 83(1), pp.280-284.
432. Wijffels, M.C., Kirchhof, C.J., Dorland, R. and Allessie, M.A., 1995. Atrial fibrillation begets atrial fibrillation: a study in awake chronically instrumented goats. *Circulation*, 92(7), pp.1954-1968.
433. Wilcken, D.E., 2010. Physiology of the normal heart. *Medicine*, 38(7), pp.336-339.
434. Windt, L.M., Wiendels, M., Dostanić, M., Bellin, M., Sarro, P.M., Mastrangeli, M., Mummery, C.L. and van Meer, B.J., 2023. Miniaturized engineered heart tissues from iPSC-derived triple cell type co-cultures to study human cardiac function. *Biochemical and Biophysical Research Communications*, 681, pp.200-211.
435. Wolf, P.A., Abbott, R.D. and Kannel, W.B., 1991. AF as an independent risk factor for stroke: the Framingham Study. *stroke*, 22(8), pp.983-988.

436. Workman, A.J., Kane, K.A. and Rankin, A.C., 2001. The contribution of ionic currents to changes in refractoriness of human atrial myocytes associated with chronic AF. *Cardiovascular research*, 52(2), pp.226-235.
437. Xie, J., Zhang, Q., Zhu, T., Zhang, Y., Liu, B., Xu, J. and Zhao, H., 2014. Substrate stiffness-regulated matrix metalloproteinase output in myocardial cells and cardiac fibroblasts: implications for myocardial fibrosis. *Acta biomaterialia*, 10(6), pp.2463-2472.
438. Xing, C., Bao, L., Li, W. and Fan, H., 2023. Progress on role of ion channels of cardiac fibroblasts in fibrosis. *Frontiers in Physiology*, 14, p.380.
439. Xu, H., Guo, W. and Nerbonne, J.M., 1999. Four kinetically distinct depolarization-activated K⁺ currents in adult mouse ventricular myocytes. *The Journal of general physiology*, 113(5), pp.661-678.
440. Yamada, N., Asano, Y., Fujita, M., Yamazaki, S., Inanobe, A., Matsuura, N., Kobayashi, H., Ohno, S., Ebana, Y., Tsukamoto, O. and Ishino, S., 2019. Mutant KCNJ3 and KCNJ5 potassium channels as novel molecular targets in bradyarrhythmias and atrial fibrillation. *Circulation*, 139(18), pp.2157-2169.
441. Yang, X., Pabon, L. and Murry, C.E., 2014. Engineering adolescence: maturation of human pluripotent stem cell-derived cardiomyocytes. *Circulation research*, 114(3), pp.511-523.
442. Yano, T., Shimoshige, S., Miki, T., Tanno, M., Mochizuki, A., Fujito, T., Yuda, S., Muranaka, A., Ogasawara, M., Hashimoto, A. and Tsuchihashi, K., 2016. Clinical impact of myocardial mTORC1 activation in nonischemic dilated cardiomyopathy. *Journal of molecular and cellular cardiology*, 91, pp.6-9.
443. Yildirim, A., Batur, M.K. and Oto, A., 2002. Hypertension and arrhythmia: blood pressure control and beyond. *Europace*, 4(2), pp.175-182.
444. Yin, X., Yin, X., Pan, X., Zhang, J., Fan, X., Li, J., Zhai, X., Jiang, L., Hao, P., Wang, J. and Chen, Y., 2023. Post-myocardial infarction fibrosis: Pathophysiology, examination, and intervention. *Frontiers in pharmacology*, 14, p.1070973.
445. Yoneda, Z.T., Anderson, K.C., Quintana, J.A., O'Neill, M.J., Sims, R.A., Glazer, A.M., Shaffer, C.M., Crawford, D.M., Stricker, T., Ye, F. and Wells, Q., 2021. Early-onset atrial fibrillation and the prevalence of rare variants in cardiomyopathy and arrhythmia genes. *JAMA cardiology*, 6(12), pp.1371-1379.
446. Yoshida, S., Miyagawa, S., Fukushima, S., Kawamura, T., Kashiwayama, N., Ohashi, F., Toyofuku, T., Toda, K. and Sawa, Y., 2018. Maturation of human induced pluripotent

- stem cell-derived cardiomyocytes by soluble factors from human mesenchymal stem cells. *Molecular Therapy*, 26(11), pp.2681-2695.
447. Young, P., Ferguson, C., Bañuelos, S. and Gautel, M., 1998. Molecular structure of the sarcomeric Z-disk: two types of titin interactions lead to an asymmetrical sorting of α -actinin. *The EMBO journal*, 17(6), pp.1614-1624.
 448. Yue, L., Xie, J. and Nattel, S., 2011. Molecular determinants of cardiac fibroblast electrical function and therapeutic implications for atrial fibrillation. *Cardiovascular research*, 89(4), pp.744-753.
 449. Zaunbrecher, R.J., Abel, A.N., Beussman, K., Leonard, A., von Frieling-Salewsky, M., Fields, P.A., Pabon, L., Reinecke, H., Yang, X., Macadangdang, J. and Kim, D.H., 2019. Cronos titin is expressed in human cardiomyocytes and necessary for normal sarcomere function. *Circulation*, 140(20), pp.1647-1660.
 450. Zech, A.T., Singh, S.R., Schlossarek, S. and Carrier, L., 2020. Autophagy in cardiomyopathies. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1867(3), p.118432.
 451. Zhang, D., Shadrin, I.Y., Lam, J., Xian, H.Q., Snodgrass, H.R. and Bursac, N., 2013. Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials*, 34(23), pp.5813-5820.
 452. Zhang, H., Shen, M. and Wu, J.C., 2020. Generation of quiescent cardiac fibroblasts derived from human iPSCs. In *Induced Pluripotent Stem (iPS) Cells: Methods and Protocols* (pp. 109-115). New York, NY: Springer US.
 453. Zhang, H., Tian, L., Shen, M., Tu, C., Wu, H., Gu, M., Paik, D.T. and Wu, J.C., 2019. Generation of quiescent cardiac fibroblasts from human induced pluripotent stem cells for in vitro modeling of cardiac fibrosis. *Circulation research*, 125(5), pp.552-566.
 454. Zhang, J., Tao, R., Campbell, K.F., Carvalho, J.L., Ruiz, E.C., Kim, G.C., Schmuck, E.G., Raval, A.N., da Rocha, A.M., Herron, T.J. and Jalife, J., 2019. Functional cardiac fibroblasts derived from human pluripotent stem cells via second heart field progenitors. *Nature communications*, 10(1), p.2238.
 455. Zhang, W., Kong, C.W., Tong, M.H., Chooi, W.H., Huang, N., Li, R.A. and Chan, B.P., 2017. Maturation of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) in 3D collagen matrix: Effects of niche cell supplementation and mechanical stimulation. *Acta Biomaterialia*, 49, pp.204-217.
 456. Zhang, Y., Su, S.A., Li, W., Ma, Y., Shen, J., Wang, Y., Shen, Y., Chen, J., Ji, Y., Xie, Y. and Ma, H., 2021. Piezo1-mediated mechanotransduction promotes cardiac

- hypertrophy by impairing calcium homeostasis to activate calpain/calcineurin signaling. *Hypertension*, 78(3), pp.647-660.
457. Zhang, Y., Yuan, B., Xu, Y., Zhou, N., Zhang, R., Lu, L. and Feng, Z., 2022. MiR-208b/miR-21 promotes the progression of cardiac fibrosis through the activation of the TGF- β 1/smad-3 signaling pathway: An in vitro and in vivo study. *Frontiers in Cardiovascular Medicine*, 9, p.924629.
 458. Zhang, Y.M., Hartzell, C., Narlow, M. and Dudley Jr, S.C., 2002. Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. *Circulation*, 106(10), pp.1294-1299.
 459. Zhao, T., Zhao, W., Chen, Y., Li, V.S., Meng, W. and Sun, Y., 2013. Platelet-derived growth factor-D promotes fibrogenesis of cardiac fibroblasts. *American Journal of Physiology-Heart and Circulatory Physiology*, 304(12), pp.H1719-H1726.
 460. Zhao, Y., Rafatian, N., Feric, N.T., Cox, B.J., Aschar-Sobbi, R., Wang, E.Y., Aggarwal, P., Zhang, B., Conant, G., Ronaldson-Bouchard, K. and Pahnke, A., 2019. A platform for generation of chamber-specific cardiac tissues and disease modeling. *Cell*, 176(4), pp.913-927.
 461. Zhong, F.J., Sun, B., Cao, M.M., Xu, C., Li, Y.M. and Yang, L.Y., 2021. STMN2 mediates nuclear translocation of Smad2/3 and enhances TGF β signaling by destabilizing microtubules to promote epithelial-mesenchymal transition in hepatocellular carcinoma. *Cancer Letters*, 506, pp.128-141.
 462. Zhou, P. and Pu, W.T., 2016. Recounting cardiac cellular composition. *Circulation research*, 118(3), pp.368-370.
 463. Zhu, C., Deng, B., Zhang, Y., Wang, Y., Xu, B. and Xiang, Y.K., 2020. Cardiomyocyte β 2-Adrenergic Receptor Plays A Protective Role in Cardiac Fibrosis. *The FASEB Journal*, 34(S1), pp.1-1.
 464. Zlochiver, S., Munoz, V., Vikstrom, K.L., Taffet, S.M., Berenfeld, O. and Jalife, J., 2008. Electrotonic myofibroblast-to-myocyte coupling increases propensity to reentrant arrhythmias in two-dimensional cardiac monolayers. *Biophysical journal*, 95(9), pp.4469-4480.
 465. Zou, J., Tran, D., Baalbaki, M., Tang, L.F., Poon, A., Pelonero, A., Titus, E.W., Yuan, C., Shi, C., Patchava, S. and Halper, E., 2015. An internal promoter underlies the difference in disease severity between N-and C-terminal truncation mutations of Titin in zebrafish. *Elife*, 4, p.e09406.

466. Zou, P., Pinotsis, N., Lange, S., Song, Y.H., Popov, A., Mavridis, I., Mayans, O.M., Gautel, M. and Wilmanns, M., 2006. Palindromic assembly of the giant muscle protein titin in the sarcomeric Z-disk. *Nature*, 439(7073), pp.229-233.
467. Zou, X., Ouyang, H., Lin, F., Zhang, H., Yang, Y., Pang, D., Han, R. and Tang, X., 2022. MYBPC3 deficiency in cardiac fibroblasts drives their activation and contributes to fibrosis. *Cell Death & Disease*, 13(11), p.948.
468. Zwi, L., Caspi, O., Arbel, G., Huber, I., Gepstein, A., Park, I.H. and Gepstein, L., 2009. Cardiomyocyte differentiation of human induced pluripotent stem cells. *Circulation*, 120(15), pp.1513-1523.