Study of Hypertrophic Cardiomyopathy Using Human Induced Pluripotent Stem Cell Derived Cardiomyocytes
CHANDRA KRISHNA PRAJAPATI

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Using Human Induced Pluripotent Stem
Cell Derived Cardiomyocytes

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE
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Study of Hypertrophic Cardiomyopathy Using Human Induced Pluripotent Stem Cell Derived Cardiomyocytes
ACADEMIC DISSERTATION
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'A person achieves 'Perfection' only if he finds joy in the work he does'

_Bhagwat Geeta_
Abstract

The human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM) technology remarkably improves in vitro disease modelling. The striking feature of hiPSC-CMs is that they retain the same genetic information as in the individual from which they are derived. Thus, hiPSCs offer a platform to study genetic diseases, screen for new potential drugs, and guide personalized medicine in the future.

Hypertrophic cardiomyopathy (HCM) is one of the most common genetic heart diseases; it has a prevalence of 1:500. HCM is primarily caused by mutations in cardiac sarcomere (~90% contractile proteins). In Finland, two founder mutations in alpha-tropomyosin (TPM1-Asp175Asn) and myosin-binding protein C (MYBPC3-Gln1061X) genes are major HCM-causing mutations, and that together account for approximately ~18% of HCM. At the tissue level, HCM is characterized by cardiomyocyte disarray, cardiac tissue fibrosis, and a thick interventricular septum or ventricular wall (15-50 mm). Clinically, symptoms vary; however, sudden death can be the first and only symptom of HCM in young adults especially among athletes. The mechanism triggering the lethal arrhythmias that cause sudden death are not understood yet. Thus, a detailed understanding of the pathophysiology of HCM should be elucidated to provide better information for the development of novel and promising treatment modalities for HCM.

The goal of this thesis was to study the electrophysiological properties of patient-specific hiPSC-CMs obtained from HCM patients carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X mutations. The first specific aim was to establish the in vitro model for HCM using hiPSC-CMs. HCM-specific hiPSC-CMs exhibited HCM phenotypes in single cells. The second aim was to use this in vitro HCM model to investigate the effects of various concentrations of adrenaline on the action potential parameters and occurrences of arrhythmias. The efficacy of bisoprolol, an antiarrhythmic agent was also examined in HCM-specific hiPSC-CMs. In addition, beat rate variabilities and repolarization variabilities were examined. The third aim was to study the relationship between two interdependent events in cardiomyocytes namely membrane potential and calcium transients by recording both parameters simultaneously.

In conclusion, in vitro disease modelling using hiPSC-CMs provides a robust platform for investigating genetic cardiac diseases. The experimental design used in this study is also suitable and beneficial for studying other cardiac diseases. In addition, simultaneously recording of membrane potential and calcium transient broadens the understanding of their interrelation and can be used to elucidate the mechanism underlying arrhythmia in more specific ways under diseased conditions.
Tiivistelmä

Ihmisen indusoidut kantasolut (hiPS-solut) ja niistä erilaistetut sydänlihassolut (hiPS-CM:t) ovat mullistaneet in vitro tautimallinnuksen. hiPS-solut sisältävät saman geneettisen information kuin se henkilö, josta solut ovat peräisin. Tämän ainutlaatuisen ominaisuutensa ansiosta hiPS-solut tarjoavat työkalun, jonka avulla voidaan tutkia perinnöllisiä sairauksia, seuloa uusia lääkkeitä, sekä tarjota yksilöllistä terveydenhoitoa tulevaisuudessa.


Johtopäätöksenä voidaan todeta, että hiPS-CM:t tarjoavat luotettavan solumallin perinnöllisten sydänairauksien tutkimusta varten. Tässä työssä käytetty koasetelma on käyttökelpoinen ja hyödyllinen myös muita sydänairauksia tutkittaessa. Tämän lisäksi V_m ja CaT -parametrien samanaikainen mitattaaminen auttaa niiden keskinäisen riippuvuuden ymmärtämisessä, ja menetelmää voidaan hyödyntää myös rytihäiriöihin johtavien mekanismien selvittämisessä.
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List of original publications

This thesis is based on the following three original publications, which are referred to as Studies I-III in the text:


# Publication was previously included in the doctoral dissertation ‘Cardiac Differentiation of Pluripotent Stem Cells and Modeling Hypertrophic Cardiomyopathy’ by Marisa Ojala at the University of Tampere, 2015.

*Authors contributed equally
List of abbreviations

\([\text{Ca}^{2+}]_i\)  Intracellular calcium level
\([\text{Ca}^{2+}]_o\)  Extracellular calcium concentration
4-AP  4-aminopyridine
AC  Adenylyl cyclase
Ach  Acetylcholine
ADP  Adenosine diphosphate
AP  Action potential
APA  Action potential amplitude
APD  Action potential duration
APD50  Action potential duration at 50\% repolarization
APD90  Action potential duration at 90\% repolarization
ARs  Adrenergic receptors
ATP  Adenosine triphosphate
AV  Atrioventricular
BPM  Bone morphogenic proteins
BRV  Beat rate variability
CaMKII  Calcium/calmodulin-dependent protein kinase II
cAMP  Cyclic adenosine monophosphate
CaT  Calcium transient
CaT90  Calcium transient at 90\% repolarization
CaTA  Calcium transient amplitude
CaTD  Calcium transient duration
CFTR  Cystic fibrosis transmembrane regulator
CICR  Calcium induced calcium release
c-MYC  Myelocytomatosis viral oncogene homolog
cPLA2  Cytosolic effector molecule phospholipase A2
CPVT  Catecholaminergic polymorphic ventricular tachycardia
cTnI  Cardiac troponin I
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>cTnT</td>
<td>Cardiac troponin T</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed after depolarization</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DHPs</td>
<td>Dihydropyridines</td>
</tr>
<tr>
<td>DI</td>
<td>Diastolic interval</td>
</tr>
<tr>
<td>dV/dt</td>
<td>Upstroke velocity</td>
</tr>
<tr>
<td>EAD</td>
<td>Early after depolarization</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>E-C</td>
<td>Excitation-contraction</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>END-2</td>
<td>Endoderm-like cells</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
</tr>
<tr>
<td>FPD</td>
<td>Field potential duration</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>HCMM-CMs</td>
<td>hiPSC-CMs with <em>MYBPC3-Gln1061X</em> mutation</td>
</tr>
<tr>
<td>HCMT-CMs</td>
<td>hiPSC-CMs with <em>TPM1-Asp175Asn</em> mutation</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hESC</td>
<td>Human embryonic stem cell</td>
</tr>
<tr>
<td>hiPSC</td>
<td>Human induced pluripotent stem cell</td>
</tr>
<tr>
<td>hiPSC-CMs</td>
<td>Human induced pluripotent stem cell-derived cardiomyocytes</td>
</tr>
<tr>
<td>I_{NCX}</td>
<td>Sodium-calcium exchanger current</td>
</tr>
<tr>
<td>I_{Ca,L}</td>
<td>L-type calcium current</td>
</tr>
<tr>
<td>I_{Ca,T}</td>
<td>T-type calcium current</td>
</tr>
<tr>
<td>ICD</td>
<td>Implantable cardioverter defibrillator</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>$I_{Cl(Ca)}$</td>
<td>Calcium dependent chloride current</td>
</tr>
<tr>
<td>$I_{Cl(cAMP)}$</td>
<td>cAMP-dependent chloride current</td>
</tr>
<tr>
<td>$I_{Cl(swell)}$</td>
<td>Swelling-activated chloride current</td>
</tr>
<tr>
<td>$I_f$</td>
<td>Funny current</td>
</tr>
<tr>
<td>$I_K(Ach)$</td>
<td>Acetylcholine activated potassium current</td>
</tr>
<tr>
<td>$I_K(ATP)$</td>
<td>ATP-sensitive potassium current</td>
</tr>
<tr>
<td>$I_K1$</td>
<td>Inward rectifier potassium current</td>
</tr>
<tr>
<td>$I_Kr$</td>
<td>Rapid activating delayed rectifier potassium current</td>
</tr>
<tr>
<td>$I_Ks$</td>
<td>Slow activating delayed rectifier potassium current</td>
</tr>
<tr>
<td>$I_{Kur}$</td>
<td>Ultra rapid activating delayed rectifier current</td>
</tr>
<tr>
<td>$I_{Na}$</td>
<td>Sodium current</td>
</tr>
<tr>
<td>$I_{P3}$</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>$I_{i}$</td>
<td>Transient inward current</td>
</tr>
<tr>
<td>$I_{io}$</td>
<td>Transient outward potassium current</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>IVS</td>
<td>Intraventricular septum</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>LIN28</td>
<td>Lin-28 homolog A</td>
</tr>
<tr>
<td>MDP</td>
<td>Maximum diastolic potential</td>
</tr>
<tr>
<td>MEA</td>
<td>Microelectrode array</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MYBPC</td>
<td>Myosin-binding protein C</td>
</tr>
<tr>
<td>Na/K-ATPase</td>
<td>Sodium/potassium-ATPase pump</td>
</tr>
<tr>
<td>NANOG</td>
<td>Nanog homeobox</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NRVT</td>
<td>Non-recovered ventricular tachycardia</td>
</tr>
<tr>
<td>NSVT</td>
<td>Non-sustained ventricular tachycardia</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>Octamer-binding transcription factor 3/4</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
</tbody>
</table>
PIP$_2$  Phosphatidylinositol 4,5-bisphosphate
PKA  Protein kinase A
PKC  Protein kinase C
PLB  Phospholamban
PLC  Phospholipase C
QES-EAD  quasi-equilibrium state EAD
RT-PCR  Real-time polymerase chain reaction
RyRs  Ryanodine receptors
SA  Sino atrial
SCD  Sudden cardiac death
SD1  Standard deviation of the instantaneous beat-to-beat interval variability
SD2  Standard deviation of the long-term beat-to-beat interval variability
SDRR  Standard deviation of RR intervals
SDSD  Standard deviation of differences between adjacent RR intervals
SERCA  Sarcoplasmic reticulum calcium-ATPase
SOX2  Sex determining region Y-box 2
SR  Sarcoplasmic reticulum
STV-50  Beat-to-beat variability at 50% repolarization
STV-90  Beat-to-beat variability at 90% repolarization
SVT  Sustained ventricular tachycardia
TEA  Tetraethylammonium
TPM  Tropomyosin
T-tubules  Transverse tubules
VF  Ventricular fibrillation
$V_m$  Membrane potential
VT  Ventricular tachycardia
Wnt  Abbreviated combination from Wingless and Integrase-1
WT-CMs  hiPSC-CMs as control
1. Introduction

Cardiac disease modelling is crucial to improve our understanding of many diseases and to discover new therapeutic approaches. Several modelling methods, including animal and computer simulation, have been used to study cardiac physiology and drug responses. Each modelling technique has its own particular advantages and disadvantages. Moreover, human-based models would be particularly useful to investigate cardiac diseases because humans and animals have differing cardiac physiologies. Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) offer the unique feature that they retain the same genetic information as the individual from which they are derived. (Sayed et al. 2016) This feature makes hiPSC-CMs superior to the conventional in vitro modelling of cardiac diseases. Furthermore, hiPSC-CMs represent an individual’s disease at the molecular and cellular level, which provides a platform to better understand the disease pathogenesis, and enables the prediction of an individual’s response to different drug treatments. Thus far, hiPSC-CMs have been demonstrated to recapitulate many genetic cardiac diseases, such as catecholaminergic polymorphic ventricular tachycardia (CPVT) (Kujala et al. 2012; Jung et al. 2012), long QT syndrome (Ma et al. 2015; Lahti et al. 2012), hypertrophic cardiomyopathy (HCM) (Lan, Andrew S Lee, et al. 2013; Han et al. 2014; Li et al. 2018), dilated cardiomyopathy (DCM) (Sun et al. 2012), and Brugada syndrome (Liang et al. 2016).

HCM is a genetic cardiac disease that has a heterogeneous clinical presentation. To date, mutations associated with HCM have been identified in at least 13 genes encoding sarcomere or sarcomere-related proteins. In Finland, mutations in alpha-tropomyosin (TPM1-Asp175Asn) and myosin-binding protein C (MYBPC3-Gln1061X) genes are major HCM-causing mutations. (Jääskelainen et al. 2013) Unexpected sudden cardiac death (SCD) has been the most devastating consequence of HCM, particularly in young patients; ventricular tachycardia (VT) and ventricular fibrillation (VF) has always been one of main causes of SCD in HCM. To understand HCM and its associated ventricular arrhythmia in a better way, protein and gene expression levels, calcium (Ca\(^{2+}\)) handling properties, and action potential (AP) recordings were investigated in HCM patient-specific hiPSC-CMs. In addition, various concentrations of adrenaline were tested, and the antiarrhythmic efficacy of bisoprolol was evaluated in these hiPSC-CMs. Furthermore, beat rate variabilities and repolarization variability were investigated. The membrane potential (\(V_m\)) and intracellular Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)) are two important aspects of cardiomyocytes. However, these parameters are generally investigated independently because incorporating two different techniques to acquire both parameters simultaneously is challenging. However, we set up systems where AP and Ca\(^{2+}\) transient (CaT) were recorded simultaneously from the same hiPSC-CMs using a conventional patch clamp system synchronized with the Ca\(^{2+}\) imaging system.

This PhD study demonstrates the establishment and utilization of HCM-specific hiPSC-CMs to study HCM diseases in detail. We strongly believe that our results improve the understanding of cardiac disease and physiology, thus creating a foundation for future studies.
2. Literature Review

2.1 Heart and cardiomyocytes

The heart is the first organ that forms in the developing foetus. Cardiomyocytes within the heart are needed for a coordinated contraction, and to ultimately pump blood efficiently. Different cardiomyocytes serve distinct and very specialized functions; however, all are electrically active. (Woodcock & Matkovich 2005)

2.1.1 Structure and function of the human heart

The circulatory system of human intergrates three basic parts/organs: the heart, the blood and, the vessels. The heart consists of two pumps, the left heart or main pump, and right heart or boost pump Figure 1A. The normal heart consists of four major cell types: myocytes, endothelial cells, smooth muscle cells, and fibroblasts. Cardiomyocytes are contracting cells, but they constitute only a minority of the cells in the heart; fibroblasts account for 40-70% of the total cells. Fibroblasts produce and maintain collagen and elastin, which serve as scaffold for myocytes. Fibroblasts are not only crucial for structural support, but also for their mechanical interaction with cardiomyocytes. (Baum & Duffy 2011) The sarcomere itself contains approximately 20 proteins. In addition, more than 20 other proteins coordinate to assemble the connection between myocytes and the extracellular matrix, and regulate cardiac contraction. In contrast to skeletal muscle, Ca$^{2+}$ entry from the extracellular space into intracellular compartment is required for excitation-contraction (E-C) coupling in cardiomyocytes. (Boron & Boulpaep 2008) Each normal heartbeat starts with a depolarization wave that originate from the sinus/sinoatrial (SA) node in the right atrium and travels over atrium and to reach the atrioventricular (AV) node. The only pathway through which the impulse spreads from the atrium to both ventricles is the His-Purkinje fibre system. The heart consists of three intrinsic pacemaking tissues capable of initiating the heartbeat: SA node, AV node, and Purkinje fibres. The pacemaker with the highest frequency, normally SA node, determines the heart rate and overrides slower pacemaker cells. An electrocardiogram (ECG) is a field potential (FP) recording of the voltage from body surfaces, which is a standard clinical tool used to record the electrical activity of heart (Figure 1B). The normal ECG consists of the following waves: P, QRS, T and U (rarely). The P wave and QRS complex represent the depolarization of atria and ventricles respectively. The T wave represents ventricular repolarization, and the U wave, which is rarely observed indicates papillary muscle repolarization. The depolarization of atria is hidden in the QRS complex. (Boron & Boulpaep 2008) The conventional body surface 12-lead ECG is “gold standard” for arrhythmia diagnosis. However, if 12-lead ECG fails to capture an arrhythmic events because of their transient and episodic occurance, 24 hour Holter ECG monitoring is used to detect various cardiac arrhythmias. The prolonged QT interval (genetic or acquired) on the ECG is associated with an increased risk of toesades de pointes and/or sudden death. (Su et al. 2013)
Figure 1. Structure of the human heart. (A) Anatomy of normal and hypertrophic heart. The human heart consists of two atria on top of heart (right atrium and left atrium), and two ventricles (right ventricle and left ventricle). These chambers are connected with valves, which allow blood to move forward, and prevent it from flowing backwards. (Image modified from cardiac inherited diseases group) (B) Components of ECG recording. It is a major diagnostic tool for assessment of heart condition. The measurement is taken at surface of skin, which reflects the electrical phenomena in heart.

2.1.2 Cardiomyocyte contraction

Cardiomyocytes are responsible for the contractile force of heart muscle. The contraction of cardiomyocytes is powered by the generation of cross-bridges between thick (myosin) and thin (actin) filaments. Within the cardiomyocytes, these myosin thick filaments and actin thin filaments are arranged in parallel, and the structure is called ‘sarcomere’. Moreover, the sarcomere is divided into the following zones: A-band (thin and thick filament overlap), I-band (consists of only thin filament), and H-band (consists of only thick filament) (Figure 2A). (Sarantitis et al. 2012)

When cardiac muscle is relaxed, actin and myosin are not interacting because myosin-binding sites to actin are blocked by rod-shaped protein called tropomyosin (TPM). The troponin complex is attached to the tropomyosin at regular interval, and troponin complex consists of three proteins: cardiac troponin I (cTnI) (I for inhibitory), which binds directly to actin, cardiac troponin T (cTnT) (T for tropomyosin), which binds troponin complex to TPM, and cardiac troponin C (cTnC) (C for Ca$^{2+}$), which binds to Ca$^{2+}$. Cardiac contraction is initiated after [Ca$^{2+}$]$_i$ rises and binds to cTnC, which cause the conformational change in TPM that exposes active site between actin and myosin. Subsequently, myosin heads interact with active actin filament sites, which induces cross-bridge formation between myosin head and active actin site (Figure 2B). The myosin binds an adenosine triphosphate (ATP) molecule and hydrolyses it to ADP and phosphate. The head of myosin containing the adenosine diphosphate ADP and phosphate is capable to interact with actin. The interaction between myosin head and actin draws actin filament along the myosin, and this process is known as power stroke. ADP is released from the myosin head, which binds to new ATP that relase the actin filament. This cycle is repeated, which allows myosin to travel along actin molecules, and progressively shorten the sarcomere. The cross-bridge releases when the [Ca$^{2+}$]$_i$ decreases, thus Ca$^{2+}$ release from cTnC, and cTnC returns to its original conformation. (Katrukha 2013; Bers 2001)
2.1.3 Cardiac ion channels, transporters, and pumps

The AP is $V_m$ waveform that is governed by complex interplay among several transmembrane ion channels and transporters (Table 1). The AP is responsible for signal propagation from cell to cell in the heart. The ion channels are highly ion selective, and this selectivity is measured in a relative sense. The majority of ion channels involved in an AP of atrial and ventricular cardiomyocytes are the same. (Nerbonne & Kass 2005) Furthermore, ion channels can be opened or closed by changes in $V_m$ (voltage-gated channels), binding of ligands (ligand-gated channels), or mechanical deformation (mechanosensitive channels). There are three main types of voltage-gated ion channels: those for sodium (Na$^+$), Ca$^{2+}$, and potassium (K$^+$). Voltage-gated Na$^+$ channels (Nav1.5, encoded by SCN5A) are largely expressed in atrial and ventricular cardiomyocytes, and mediate the influx of Na$^+$ ions ($I_{Na}$). (Nerbonne & Kass 2005) The Nav1.5 consists of four homologous domains (I-IV), each of which has six transmembrane segments (TM1 to TM6). The fast activation of Nav1.5 channels results in fast depolarization; the activation voltage of Nav1.5 is quite negative (~ -55 mV). In addition, Nav1.5 also inactivates quickly and is voltage dependent. Thus at a $V_m$ near the plateau phase of AP, ~ 90% of Nav1.5 channels are inactivated, i.e. they are in a non-conducting state. There is also a presence of sustained component of Na$^+$ current (persistent current) that also play a role in determining the APD (Coppini et al. 2013). Mutations in SCN5A are linked with several cardiac diseases.
including long QT syndrome and Brugada syndrome. (Nerbonne & Kass 2005; Bartos et al. 2015) In the absence of extracellular Na⁺, some Na⁺ channels allow Ca²⁺ influx; however, this influx is several hundred times less efficient than Na⁺ influx because the selectivity of Na⁺ channels for Na⁺ relative to Ca²⁺ (PNa/PCa) is > 3000. The Nav1.5 are modulated by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and protein kinase C (PKC). (Bers 2001)

Both voltage-gated T-type (Cav3.1) and L-type (Cav1.2) Ca²⁺ channels are expressed in the heart. The Cav3.1 channels are low voltage activated channels; they activate at more negative V_m (~ -50 mV), and are functionally expressed during development, and downregulated in adult cardiomyocytes. In addition, Cav3.1 channels play an important role in pacemaker activity in the SA node. The high voltage activated Cav1.2 channels are abundantly expressed, and are the primary pathway of extracellular Ca²⁺ influx resulting in Ca²⁺-induced Ca²⁺ release (CICR). These channels activate at approximately -20 mV. The inactivation of Cav1.2 channels is slower than that of Cav3.1 channels, and both are V_m- and Ca²⁺-dependent. Furthermore, the properties and Ca²⁺ current densities (I_Ca,L) of Cav1.2 in cardiomyocytes obtained from different regions of the myocardium are quite similar. (Bers 2001; Nerbonne & Kass 2005) The Cav1.2 has similar topology as Nav1.5, and consists of α, α2/δ, β, and, in some tissues, γ subunits. CaV1.2 channels are sensitive to dihydropyridines (DHPs); most DHPs act as I_Ca,L blockers (for example, nifedipine, nimodipine) whereas some DHPs act as I_Ca,L agonists (for example, Bay K 8644). Furthermore, Cav1.2 channels are stimulated by PKA, which increases both the amplitude and the inactivation rate. (Bers 2001)

Voltage-gated K⁺ channels are primarily responsible for AP repolarization. The K⁺ channels are the most diverse channels; they produce an outward current at physiological V_m, and tend to drive V_m toward the equilibrium potential (E_K). There are two broad classes of repolarizing K⁺ currents based on time- and V_m-dependent properties, and pharmacological sensitivities; these are transient outward K⁺ currents (I_o), and delayed outward rectifying K⁺ currents (I_K). The I_o is composed of two different components- Ca²⁺-independent K⁺ current (I_o,1) and Ca²⁺-dependent chloride (Cl⁻) current (I_o,2). Furthermore, I_o,1 is sensitive to 4-aminopyridine (4-AP), and unaffected by extracellular Ca²⁺ whereas I_o,2 is 4-AP insensitive, Ca²⁺-dependent Cl⁻ current (I_{Cl(Ca)}) rather than a K⁺ current. (Nerbonne & Kass 2005; Bers 2001) The I_{Cl(Ca)} channels do not have intrinsic V_m-dependence or rectification, but are activated by the [Ca²⁺]i during E-C coupling. The I_{Cl(Ca)} also refers to I_o,2, peak at V_m of +20 mV to +40 mV, and decreases at a more positive V_m. In human atrial and ventricular cardiomyocytes, the presence of I_o,2 is not clearly demonstrated. The current is divided into a faster inactivating current (I_{o,1}) and slower inactivating current (I_{o,2}); however, both activate rapidly. Moreover, I_{o,1} is mediated by Kv4.2 and/or Kv4.3 whereas I_{o,2} is mediated by Kv1.4. The Kv4.3 is abundantly expressed in epicardium, and is responsible for shorter AP duration (APD), but Kv1.4 is expressed to a much lesser extent. (Amin et al. 2010)

I_K is the major current responsible for AP repolarization. There are at least three delayed rectifier K channels in the heart: rapid (I_Kr), slow (I_Ks), and ultra-rapid (I_Kur). These currents display different kinetics and pharmacological properties, and are encoded by separate genes. The human-ether-a-go-go-related gene (hERG) encodes the α-subunit (Kv11.1) of the channel.
carrying \( I_{Kr} \). \( K_{V11.1} \) channels activate at \( \sim -30 \) mV, but this activation is not rapid upon depolarization, whereas inactivation is fast. \( I_{Kr} \) is blocked by methanesulfonanilides such as doxetilide, E-4031, almokalant and sotalol. The elevation of intracellular cAMP levels regulates the \( I_{Kr} \) channels via PKA-mediation. Furthermore, PKA activation reduces the \( I_{Kr} \) current amplitude. (Tamargo et al. 2004; Amin et al. 2010). \( I_{Kr} \) activates slowly at \( V_m \) positive to -30 mV. The pore-forming subunit is \( K_{V7.1} \), which is encoded by KCNQ1. \( I_{Kr} \) is resistant to methanesulfonanilide, but is blocked by chromanols (293B, HMR-1556), indapamide, thiopentone, propofol and benzodiazepines (L-768,673, L-735,821 or L-7). \( I_{Kr} \) is strongly regulated by PKA and PKC in a \( V_m \)- and temperature-dependent manner. (Amin et al. 2010; Tamargo et al. 2004) The \( I_{Kur} \) activates more rapidly than either \( I_{Kr} \) or \( I_{Ks} \). \( I_{Kur} \) is expressed in atrial cardiomyocytes but not in ventricular cardiomyocytes. \( K_{V1.5} \) encodes the \( \alpha \)-subunit of the \( I_{Kur} \) channel. \( I_{Kur} \) is relatively insensitive to tetraethylammonium (TEA), \( Ba^{2+} \) and the methanesulfonanilide group, but is highly sensitive to 4-AP (\( \mu M \)). (Amin et al. 2010; Bers 2001) Inward rectification means the channel (Kir2.1), encoded by the KCNJ2 gene conducts the inward current (\( I_{K1} \)) better than the outward current. \( I_{K1} \) is a strong rectifier \( K^+ \) current that works over a limited range of \( V_m \), and keep the resting \( V_m \) close to \( E_K \). Physiologically, the outward \( I_{K1} \) current is important because the resting \( V_m \) never reach beyond the reversal potential of \( I_{K1} \), and as a result the inward movement of \( K^+ \) via \( I_{K1} \) is not possible. \( I_{Kr} \) is blocked by extracellular \( Ba^{2+} \), and intracellular \( Cs^+ \). Blocking \( I_{K1} \) results in depolarized \( V_m \) and longer APD. \( I_{K1} \) can be suppressed by PKA-mediated phosphorylation of the channel. (Tamargo et al. 2004; Amin et al. 2010; Bers 2001)

Hyperpolarization-activated cyclic nucleotide-gated channels (HCN) underlies the cardiac pacemaker activity. The current is named ‘funny’ (\( I_f \)) because it activates slowly upon hyperpolarization, which is in contrast to earlier described currents those are activated upon depolarization. \( I_f \) is a mixed \( Na^+/K^+ \) current, but other monovalent cations (Li, Cs, Rb) are almost impermeant. (Bers 2001) The \( V_m \)-dependent activation of \( I_f \) differs greatly in different tissues; \( V_m \)-dependent activation range overlaps the range of \( V_m \) in SA node (i.e. -40 mV to 60 mV) whereas \( V_m \)-dependent activation is further than the resting \( V_m \) in ventricular cardiomyocytes. Moreover, ivabradine is an \( I_f \) blocker, and \( I_f \) activation is accelerated when intracellular cAMP levels are increased. (Bers 2001)

There are also various ligand-gated channels in cardiomyocytes. Acetylcholine (Ach)-activated \( K^+ \) channels (\( K_{Ach} \)) are activated by muscarinic agonists, such as acetylcholine. \( K_{Ach} \) are composed of a tetrameric complex of two Kir3.1 and two Kir3.4 subunits. These channels are predominately presented in atrial cardiomyocytes, SA and AV nodes. Currents through \( K_{Ach} \) (\( I_{K(Ach)} \)) can also be activated by other agonists couple to G-proteins, such as adenosine, ATP, and PIP2, and the currents are inhibited by intracellular acidification and several antiarrhythmic drugs. \( I_{K(Ach)} \) exhibits desensitization upon sustained exposure to Ach, which results in a gradual decrease in current. (Bers 2001) ATP-sensitive \( K^+ \) channels (\( K_{ATP} \)) are activated by falling intracellular ATP levels ([ATP]i) and rising ADP level [ADP]i. Cardiac \( K_{ATP} \) channels are composed of \( \alpha \)-subunits (Kir6.2) and SUR2A subunits. The Kir6.2 mediates inhibition by [ATP]i, whereas the SUR2A subunit mediates sensitivity to [ADP]i. Thus, current through \( K_{ATP} \) (\( I_{K(ATP)} \)) is inhibited by [ATP], and activated by [ADP], and thus the channel activity is regulated by the [ATP]/[ADP]i ratio i.e. energetic state of cells. When the intracellular ATP
level fall down during local ischemia, $K_{(ATP)}$ activation can shorten APD and also prevent excitability. $K_{ATP}$ channels are blocked by sulfonylureas (i.e., glibenclamide, glicazide, glipizide, glimepiride, tolbutamide), and glinides (repaglinide, nateglinide). (Bers 2001; Tamargo et al. 2004) The cAMP-dependent Cl\(^-\) channels (Cl(cAMP)) in the heart are responsible for the autonomic regulation of APD, and the current through Cl(cAMP) (I_{Cl(cAMP)}) is carried by a cardiac variant of the cystic fibrosis transmembrane regulator (CFTR). PKA-dependent phosphorylation activates Cl(cAMP). I_{Cl(cAMP)} is inhibited by anthracene-9-carboxylic acid (9-AC), niflumic acid, and glibenclamide. (Bers 2001) Swelling-activated Cl\(^-\) channels (Cl(swell)) are not only stimulated by osmotic and hydrostatic increases in cell volume, but also by agents, that alter the membrane tension and by direct mechanical stretching. The current through Cl(swell) (I_{Cl(swell)}) is outwardly rectifying (either physiologic or symmetric Cl\(^-\) gradient), which shortens the APD, depolarize the Vm and works to decrease the cell size. These channels can be blocked by niflumate or tamoxifen. (Baumgarten & Clemo 2003) I_{Cl(swell)} is time-independent over all of the physiologic Vm, but is partially inactivated at positive potentials. I_{Cl(swell)} is independent of intra- or extracellular Ca\(^{2+}\). Thus, it can be elicited in the absence of extracellular Ca\(^{2+}\) and after the strong buffering of $[Ca^{2+}]$. (Baumgarten & Clemo 2003; Bers 2001)

The Na\(^{+}/Ca^{2+}\) exchanger (NCX) is the major ionic pump in cardiomyocytes involved in Ca\(^{2+}\) regulation, especially the sarcoplasmic reticulum (SR) Ca\(^{2+}\) content, and thus it influences the cardiomyocyte contractility. NCX transports 3 Na\(^+\) ions in exchange for 1 Ca\(^{2+}\) ion. In addition, NCX can perform bidirectionally; the direction and amplitude of the NCX current (I_{NCX}) depends on Vm and intra- and extracellular Na\(^+\) and Ca\(^{2+}\) concentrations. In forward mode, NCX extrude 1 Ca\(^{2+}\) from cardiomyocytes in exchange of 3 Na\(^+\) influx. Conversely, in reverse mode, NCX extrude 3 Na\(^+\) in exchange for 1 Ca\(^{2+}\) influx. (Ottolia et al. 2013) NCX utilizes energy in the trans-sarcolemmal Na\(^+\) gradient to move ions into or out of the cells. Thus, the Na\(^+\)/K\(^+\)-ATPase pump is essential for re-establishing and maintaining this gradient. Na\(^+\)/K\(^+\)-ATPase transports 3 Na\(^+\) out and 2 K\(^+\) into the cells using the energy of ATP molecules. By pumping more positive ions out of cells than into cells, this Na/K-ATPase pump creates the negative potential within cells. The free energy of intracellular ATP hydrolysis ($\Delta G_{ATP}$) provides the energy required for active Na\(^+\) and K\(^+\) transport across the cell membrane. Na/K-ATPase also has reversal potential (-180 mV under normal condition) depending on both intracellular and extracellular Na\(^+\) and K\(^+\) concentrations and $\Delta G_{ATP}$. (Glitsch & Tappe 1995; Bielen et al. 1991)
**Table 1.** Cardiac ion channels

<table>
<thead>
<tr>
<th>Current</th>
<th>α-subunit</th>
<th>Gene</th>
<th>AP phase</th>
<th>blocking agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Voltage-gated ion channels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{Na}$</td>
<td>Nav1.5</td>
<td><em>SCN5A</em></td>
<td>Phase 0</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>$I_{to,fast}$</td>
<td>Kv4.3</td>
<td><em>KCND3</em></td>
<td>Phase 1</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>$I_{to,slow}$</td>
<td>Kv1.4</td>
<td><em>KCNA4</em></td>
<td>Phase 1</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>$I_{Ca,L}$</td>
<td>Cav1.2</td>
<td><em>CACNA1C</em></td>
<td>Phase 2</td>
<td>Cations (Mg$^{2+}$, Ni$^{2+}$, Zn$^{2+}$) Dihydropyridines Phenylalkylamines, Benzothiazepines</td>
</tr>
<tr>
<td>$I_{Ca,T}$</td>
<td>Cav3.1</td>
<td><em>CACNA1G</em></td>
<td>Phase 2</td>
<td>similar as $I_{Ca,L}$ (potency may differ)</td>
</tr>
<tr>
<td></td>
<td>Cav3.2</td>
<td><em>CACNA1H</em></td>
<td>Phase 2</td>
<td></td>
</tr>
<tr>
<td>$I_{Kur}$</td>
<td>Kv1.5</td>
<td><em>KCNA5</em></td>
<td>Phase 1</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>$I_{Kr}$</td>
<td>Kv11.1</td>
<td><em>KCNH2</em></td>
<td>Phase 3</td>
<td>E-4031</td>
</tr>
<tr>
<td>$I_{Ks}$</td>
<td>Kv7.1</td>
<td><em>KCNQ1</em></td>
<td>Phase 3</td>
<td>Chromanol-293B</td>
</tr>
<tr>
<td>$I_{K1}$</td>
<td>Kir2.1</td>
<td><em>KCNJ2</em></td>
<td>Phase 3 &amp; 4</td>
<td>Ba$^{2+}$</td>
</tr>
<tr>
<td>$I_f$</td>
<td>HCN1-4</td>
<td></td>
<td>Phase 4</td>
<td>Cs$^+$</td>
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<tr>
<td><strong>Ligand gated channel</strong></td>
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</tr>
<tr>
<td>$I_{K(ACh)}$</td>
<td>Kir3.1:3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{K(ATP)}$</td>
<td>Kir6.2</td>
<td></td>
<td></td>
<td>Glibenclamide</td>
</tr>
<tr>
<td>$I_{Cl(Ca)}$</td>
<td>-</td>
<td></td>
<td></td>
<td>Niflumate</td>
</tr>
<tr>
<td>$I_{Cl(cAMP)}$</td>
<td>CFTR</td>
<td></td>
<td></td>
<td>9-Aminoacridine</td>
</tr>
<tr>
<td><strong>Mechanosensitive channels</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$I_{Cl(Swell)}$</td>
<td>CIC-3</td>
<td></td>
<td></td>
<td>4,4’-diisothiocyanatostilbene</td>
</tr>
<tr>
<td>$I_{NS(stretch)}$</td>
<td>?</td>
<td></td>
<td></td>
<td>Gd</td>
</tr>
</tbody>
</table>

$I_{Na}$: Na$^+$ current, $I_{to,fast/slow}$: transient outward K$^+$ current (fast and slow recovery from inactivation), $I_{Ca,L}$: L-type Ca$^{2+}$ current, $I_{Ca,T}$: T-type Ca$^{2+}$ current, $I_{Kur}$: ultra-rapidly activating delayed outward rectifying K$^+$ current, $I_{Kr}$: rapid activating delayed outward rectifying K$^+$ current, $I_{Ks}$: slow activating delayed outward rectifying K$^+$ current, $I_{K1}$: inward rectifier K$^+$ current, $I_f$: funny current. (Grant 2009; Bers 2001)
2.1.4 Cellular cardiac electrophysiology

Electrical activity of cardiac cells is determined by the net influx/efflux of ions through voltage-gated ion channels, ionic pumps, transporters, and exchangers. Voltage-gated ionic channels are controlled by transmembrane potential, i.e. the opening and closing of gates is controlled by $V_m$. The normal cardiac AP consists of different phases (Figure 3). Different currents predominate during each phase of AP. The fast $I_{Na}$ current is responsible for the upstroke (phase 0) of AP, which activates and inactivates very quickly. After rapid depolarization, $I_{to}$ current is activated to ensure initial repolarization (phase 1). Inward $I_{Ca,L}$ current is activated for sustaining of the AP plateau (phase 2) that triggers the release of intracellular Ca$^{2+}$ from sarcoplasmic reticulum required for cardiac contraction (E-C coupling). As the $V_m$ further move to more negative $V_m$, two main types of repolarizing K$^+$ currents ($I_{Kr}$ and $I_{Ks}$) are activated to enable terminal repolarization (phase 3). The $I_{K1}$ current is activated during and following final repolarization phase to ensure terminal repolarization and a stable $V_m$ (phase 4). Furthermore, NCX continues to extrude intracellular Ca$^{2+}$ stored during AP in exchange for extracellular Na$^+$, thus creating net inward current. In addition, Na/K-ATPase is activated to transport accumulated intracellular Na$^+$ out of the cell at the expense of ATP to maintain ionic homeostasis. (Grant 2009)

Because of differences in the presence and expression of different ion channels, the shape, duration, and properties of AP vary among ventricular-, atrial- and nodal- cardiomyocytes. Ventricular cardiomyocytes maintain a slightly more hyperpolarized resting $V_m$ (~ -85 mV) compared to atrial cardiomyocytes (~ -80 mV), and one possible reason is higher expression of $I_{K1}$ in ventricular cardiomyocytes. In addition, the plateau phase is longer in ventricular cardiomyocytes than in atrial cardiomyocytes because of lower density of K$^+$ currents activated during the notch phase. Moreover, upstroke velocity (dV/dt) is faster, and repolarization occurs at a faster rate in ventricular cardiomyocytes than in atrial cardiomyocytes. (Bartos et al. 2015) Unlike atrial- and ventricular cardiomyocytes, nodal cardiomyocytes consist of funny current and lack $I_{K1}$, thus exhibiting self-beating properties. In addition, nodal cells achieve AP mainly by Ca$^{2+}$ currents ($I_{Ca,T}$ and $I_{Ca,L}$). (Amin et al. 2010; Nerbonne & Kass 2005; Bartos et al. 2015)

AP heterogeneity exhibits across the myocardial wall from the endocardium, midmyocardium to the epicardium. Epicardial cells have prominent phase 1 and the shortest APD whereas midmyocardial cells have the longest APD. Furthermore, the average duration of ventricular AP is represented by the QT interval in ECG. (Grant 2009)
Figure 3. Action potential (AP) profiles and ionic currents involved. Inward depolarizing currents and outward repolarizing currents underlying atrial and ventricular action potential. Ventricular AP exhibit a prominent plateau phase; atrial AP are more triangular in shape. Phase 0, rapid depolarization; phase 1, rapid early repolarization phase; phase 2, slow repolarization phase (‘plateau’ phase); phase 3, rapid late repolarization phase; phase 4, resting membrane potential. Image modified from (Ravens & Cerbai 2008).

2.1.5 Calcium handling in cardiomyocytes

Ca$^{2+}$ plays a crucial role in cardiac E-C coupling; Ca$^{2+}$ activates the myofilaments during cardiac systole causing contraction while Ca$^{2+}$ dissociates from myofilaments during diastole and lead to relaxation (Figure 4). During an AP, a small amount of Ca$^{2+}$ enters into cardiomyocytes via I$_{Ca,L}$, which triggers the greater amount of Ca$^{2+}$ release from SR through ryanodine receptors (RyRs) into cytosol- the process known as CICR. This process increases [Ca$^{2+}$]$_i$ from diastolic value of 0.1 µM/L to systolic value of approximately 1 µM/L. In addition, Ca$^{2+}$ may enter cells via NCX to increase the [Ca$^{2+}$]$_i$. Increased [Ca$^{2+}$]$_i$ bind troponin C leading to conformational changes and the contraction of cardiomyocytes. (Neef & Maier 2013) The contraction force exhibits a nonlinear relationship with [Ca$^{2+}$]$_i$, and depends on the CaT amplitude (CaTA) and duration (CaTD), and on sensitivity of myofilaments to Ca$^{2+}$. The sensitivity of myofilaments to Ca$^{2+}$ is enhanced by myofilament stretching, caffeine, and inotropic drugs, whereas β-adrenergic activation, acidosis and elevated phosphate and magnesium concentration reduce the sensitivity. (Bers 2002) There are two main mechanisms involved in reducing an elevated [Ca$^{2+}$]$_i$ to facilitate the relaxation of cardiomyocytes. The
The majority (~70%) of cytoplasmic Ca\(^{2+}\) decrease is caused by reuptake into SR via SR Ca\(^{2+}\)-ATPase (SERCA2a), and a minority (~28%) of Ca\(^{2+}\) is extrusion through NCX functioning in forward mode, i.e., removing 1 Ca\(^{2+}\) in exchange for 3 Na\(^+\). The remaining 2% Ca\(^{2+}\) release uses the Ca\(^{2+}\) extrusion pathway (mitochondrial Ca\(^{2+}\)-uniport and sarcolemmal CA\(^{2+}\)-ATPase). (Neef and Maier 2013) The PKA and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) play important roles in controlling the E-C coupling. The β-adrenergic stimulation activates PKA via cAMP, and accelerates SR Ca\(^{2+}\) uptake, and increases SR Ca\(^{2+}\) loading. (Bers 2002; Neef & Maier 2013)

**Figure 4.** Schematic diagram showing excitation-contraction coupling in cardiomyocytes. 1. Depolarization of membrane potential (V\(_m\)), 2. Opening of L-type Ca\(^{2+}\) channels and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) in reverse mode, 3. Opening of ryanodine receptors (RyR2) 4. Release of Ca\(^{2+}\) from sarcoplasmic reticulum (SR) 5. Binding of Ca\(^{2+}\) to troponin C in myofilament 6. Re-uptake of Ca\(^{2+}\) into SR via SR Ca\(^{2+}\) ATPase-2a (SERCA2a), and 7. Ca\(^{2+}\) extrusion out of cytosol through NCX and membrane Ca\(^{2+}\) ATPase 8, and mitochondrial Ca\(^{2+}\) uptake. Image modified from (Bers 2002).

The major factor controlling the amount of SR Ca\(^{2+}\) release, and CaTA is SR Ca content. The SR Ca\(^{2+}\) content is determined by the balance between reuptake of Ca\(^{2+}\) via SERCA and SR Ca\(^{2+}\) release through RyR. (Díaz et al. 2005) Ca\(^{2+}\) buffering is a mechanism to control Ca\(^{2+}\) persistence in the cytosol, and thereby control the many Ca\(^{2+}\) dependent intracellular processes. In cardiomyocytes, [Ca\(^{2+}\)]\(_i\) is highly buffered, and the major Ca\(^{2+}\) buffers are troponin and SERCA. (Briston et al. 2014) Furthermore, phosphorylation of troponin decrease its affinity for Ca binding (Robertson et al. 1982), but phosphorylation of phospholamban (PLB) increases...
the affinity of SERCA for Ca$^{2+}$ (Tada et al. 1974). The buffering decreases the rate constant of decay of cytoplasmic Ca$^{2+}$. Ca$^{2+}$ buffering depends on [Ca$^{2+}$_i]; the buffering power is greater at low [Ca$^{2+}$_i], whereas it decreases at higher levels as buffers tend to saturate. Other factors may also affect buffering power despite unchanged [Ca$^{2+}$_i]. For example, in genetic cardiac disease, such as HCM caused by mutation either in troponin or in TPM results in the alternation of Ca$^{2+}$ binding to cardiac myofibrils. (Bottinelli et al. 1998) Although atrial and ventricular cardiomyocytes have many shared properties, some differences in ultrastructure exist between them, such as a lack of transverse tubules (t-tubules) (‘z-tubules’ instead), RyR distribution, and I$_{ca}$ location in atrial cardiomyocytes, which gives them different Ca$^{2+}$ handling properties. In addition, other proteins involved in Ca$^{2+}$ handling such as calsequestrin, junctin and triadin are expressed at lower levels in atrial cells. In ventricular myocytes, SERCA activity is substantially regulated by phospholamban whereas atrial cells utilise both phospholamban and a smaller proteolipid called sarcolipin, to regulate SERCA activity. (Bootman et al. 2011) SR Ca$^{2+}$ content is governed by Ca$^{2+}$ fluxes across the sarcolemma, Ca$^{2+}$ reuptake into SR, and amount of Ca$^{2+}$ buffering proteins within SR. However, Ca$^{2+}$ handling and Ca$^{2+}$ buffering properties are altered in atrial cardiomyocytes than ventricle cardiomyocytes from rats. (Bootman et al. 2011) The Ca$^{2+}$ buffering power is higher in atrial cardiomyocytes mainly because of the higher expression of SERCA protein and increased affinity of SERCA for Ca$^{2+}$. Although buffering power is increased in atrial cardiomyocytes, Ca$^{2+}$ reuptake by SERCA is faster, which is likely because of an increased SERCA to PLB ratio. (Walden et al. 2009)

2.1.6 Abnormalities in action potentials

Abnormalities in APs are AP aberration, and they are usually categorized by the take-off potential. If AP has already returned to diastolic $V_m$ prior to depolarization, it is referred as delayed after depolarization (DAD) (Figure 5A). However, if the take-off potential is during phase 2 or phase 3 of AP, it is known as early after depolarization (EAD) (Figure 5B-C).

The cellular and SR Ca$^{2+}$ overload increase the [Ca$^{2+}$_i], which activates Ca$^{2+}$-dependent transient inward currents (I$_{s}$), such as I$_{Na/Ca}$ and I$_{C/L(Ca)}$, and as a consequence, DAD occurs. (Zygmont et al. 1998) An individual Ca$^{2+}$ spark usually does not produce sufficient I$_{s}$ (mainly via I$_{Na/Ca}$) to cause measurable DAD. However, localized Ca$^{2+}$ sparks may merge to form a larger Ca$^{2+}$ wave within cells, and cause the depolarization of the $V_m$. (Wagner et al. 2015) Furthermore, the time course and amplitude of the DAD depend on the (i) time course and amplitude of an integrated Ca$^{2+}$ spark and (ii) the sensitivity of the resting $V_m$ to change in [Ca$^{2+}$_i] (i.e., Ca$^{2+}$-voltage coupling gain) (Maruyama et al. 2010)

Prolongation of the APD is frequently associated with the occurrence of EADs. EADs occurring at plateau phase are attributable to the reactivation of I$_{ca}$; $V_m$ falls into the range of I$_{ca}$ window current, thus allowing I$_{ca}$ to recover and cause depolarization. However, EADs occurring at late phase (phase-3 EAD) i.e., below -40 mV are less likely to happen due to the reactivation of Ca$^{2+}$ channels. This is because Ca$^{2+}$ channels recovered from inactivation would not activate at such negative $V_m$. However, phase-3 EADs may occur because of Ca$^{2+}$-activated currents. (Bers 2001)
2.1.7 Interrelation between AP and calcium handling

Ca$^{2+}$ is the major ion regulating the E-C coupling in cardiomyocytes. Thus $[\text{Ca}^{2+}]_i$ measurements are crucial for understanding not only cardiac contraction and relaxation, but also the mechanism of arrhythmias. The $[\text{Ca}^{2+}]_i$ can affect the shape and APD via Ca$^{2+}$-sensitive ionic currents such as $I_{\text{Ca}}$, $I_{\text{Na/Ca}}$, and $I_{\text{Cl(Ca)}}$ thereby modifying the electrophysiological properties, e.g., refractoriness and membrane depolarization rate. (Bers 2002) Conversely, $V_m$ can influence the Ca$^{2+}$ spark and waves (Sato et al. 2014). Therefore, $V_m$ and Ca$^{2+}$ dynamics are interdependent and bi-directionally coupled. Bi-directional coupling of $V_m$ and $[\text{Ca}^{2+}]_i$ ($V_m \leftrightarrow [\text{Ca}^{2+}]_i$) is defined by the facts that (i) $V_m$ directly influence the activity of Ca$^{2+}$ handling mechanisms ($V_m \rightarrow [\text{Ca}^{2+}]_i$ coupling), and (ii) Ca$^{2+}$ determine the $V_m$ through Ca$^{2+}$-dependent ion currents and transporters ($[\text{Ca}^{2+}]_i \rightarrow V_m$). In addition, extracellular Ca$^{2+}$ concentration $[\text{Ca}^{2+}]_o$ can also alter the gating of all voltage dependent ion channels. (Bers 2001) High $[\text{Ca}^{2+}]_o$ moves channel activation to a more positive $V_m$ thus reducing the excitability whereas low $[\text{Ca}^{2+}]_o$ moves channel activation to a more negative $V_m$ increasing the excitability. (Bers 2002)

However, disturbances/instabilities in bi-directional coupling between $V_m$ and $[\text{Ca}^{2+}]_i$ is one of the main reasons for the generation of alternans; APD alternans lead to the generation of CaT alternans and vice versa. (Edwards & Blatter 2014; Gaeta & Christini 2012) The APD influences the CaT through the magnitude of SR Ca$^{2+}$ release, which is proportional to size of $I_{\text{Ca}}$ (‘graded release’). The $I_{\text{Ca}}$ itself depends on the length of the preceding diastolic interval (DI); a shorter DI results in smaller Ca$^{2+}$ release and vice versa. In other words, the prolongation of APD causes a smaller Ca$^{2+}$ release on following AP, and is defined as ‘positive’ $V_m \rightarrow [\text{Ca}^{2+}]_i$. Moreover, $V_m \rightarrow [\text{Ca}^{2+}]_i$ coupling is generally positive, meaning that a longer APD occurs in parallel with a CaTA. (Edwards & Blatter 2014; Gaeta & Christini 2012) Positive
coupling between APD and CaTD is also referred to as ‘in-phase’ or ‘concordant’, whereas ‘negative’ coupling results in ‘out-of-phase’ or ‘discordant’ alternans at the single cell level. On the other hand, changes in [Ca\(^{2+}\)] can be positively or negatively coupled with V\(_m\) ([Ca\(^{2+}\)]\(_\rightarrow\)V\(_m\) coupling) depending on which of the Ca\(^{2+}\)-dependent ion currents or transporters dominate over another (i.e., opposing effects of NCX and Ca\(^{2+}\)-induced inactivation of I\(_{Ca,L}\)). A positive [Ca\(^{2+}\)]\(_\rightarrow\)V\(_m\) coupling occurs when the large CaT causes a prolongation of APD by potentiating the inward I\(_{\text{NCX}}\) to a greater extent than reducing I\(_{Ca,L}\) through Ca\(^{2+}\)-dependent inactivation. In contrast, negative [Ca\(^{2+}\)]\(_\rightarrow\)V\(_m\) coupling occurs when effect of Ca\(^{2+}\)-induced inactivation of I\(_{Ca,L}\) dominates over increased I\(_{\text{NCX}}\), thus causing the larger CaT to shorten the APD. (Edwards & Blatter 2014; Gaeta & Christini 2012) The APD and CaT alternans are strongly correlated in time and magnitude in both atrial and ventricular cardiomyocytes. Of note, CaT alternans can develop in the absence or presence of APD alternans, and V\(_m\) \(\rightarrow\) [Ca\(^{2+}\)] coupling might play a more prominent role in atrial than in ventricular cardiomyocytes. (Kanaporis & Blatter 2015)

### 2.1.8 Adrenergic receptors in cardiomyocytes

Adrenergic receptors (ARs) are G-protein-coupled receptors (GPCRs), and activated by the binding of ligands such as hormones or neurotransmitters and they have essential roles in heart function (Figure 6). These receptors participate in the regulation of many important physiological functions, and serve as pharmacological targets in disease conditions. The human heart expresses of two ARs: β-ARs and α-ARs, which constitute approximately 90% and 10% respectively of the total ARs. (O'Connell et al. 2014) The α-ARs are classified into two groups, α\(_1\)-AR and α\(_2\)-AR, which are further subdivided into three groups namely α\(_1\)-ARs: α\(_1\)A, α\(_1\)B and α\(_1\)D, and α\(_2\)-ARs: α\(_2\)A, α\(_2\)B and α\(_2\)C. Cardiac myocytes only express α\(_1\)-ARs; however, their functional role in cardiac cells remain unclear. Upon α\(_1\)-ARs activation, G\(_q\) activates phospholipase C (PLC) that breaks phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). DAG activates PKC and IP\(_3\) either activates or modulates Ca\(^{2+}\) release. PKA activation stimulates Na\(^+\)/H\(^+\) exchange to extrude protons, which increase in the intracellular pH (contributes to increase myofilament Ca\(^{2+}\) sensitivity) and the Na\(^+\) level (increases in Ca\(^{2+}\) transient via NCX). (Bers 2001) In addition, α\(_1\)-ARs are involved in regulation of growth. After α\(_1\)-ARs, no change in AP configuration was observed in ventricular cardiomyocytes (Jakob et al. 1988), but a slight decrease in APD was observed in atrial cardiomyocytes (Jahnel et al. 1992) in an early study. Furthermore, β-ARs are divided into three subtypes namely β\(_1\)-, β\(_2\)- and β\(_3\)-AR. The β\(_1\)-ARs are primarily found in the human heart and account for 75-80% of total β-ARs. The β\(_2\)-AR comprises 15-18% of cardiac β-ARs, and β\(_3\)-ARs are minimally (2-3%) expressed in the heart. Activation of β-ARs (mainly β\(_1\)-ARs but to a lesser extent β\(_2\)-ARs) causes an increase in cardiac contractility (inotropy), frequency (chronotrophy), rate of relaxation (lusitropy) and impulse conduction through the AV node (dromotropy). (Lymeropoulos A 2013) In the human heart, β\(_1\) and β\(_2\)-adrenoceptors coexist; the amount of β\(_2\)-adrenoceptors is higher in the atria (approximately 30% of the total β-adrenoceptor population) than in the ventricular myocardium.
Stimulation of both $\beta_1$- and $\beta_2$-ARs causes a maximal increases in contractile force in the atria; in the ventricular myocardium, however, only $\beta_1$-ARs stimulation maximally increases contractile force, and $\beta_2$-ARs stimulation evokes only submaximal increases. (Khamssi & Brodde 1990)

Epinephrine and norepinephrine are the primary agonists for all $\beta$-ARs. Compelling differences exist in the signalling pathways and cellular responses among $\beta$-ARs. (Brodde & Michel 1999) $\beta_1$-AR binds to G stimulatory ($G_s$) protein, and the $G_s$ subunit of $G_s$ protein activates adenylyl cyclase (AC), which generates the second messenger cAMP, which in turn activates cAMP-dependent PKA. Activated PKA then phosphorylates cTnI, $I_{Ca,L}$ and PLB increases the contractility. (Madamanchi 2007) Along with $G_s$, $\beta_2$-AR can couple to G inhibitory ($G_i$) protein and release the activated $G_i\alpha$ subunit that inhibits AC activity and $G_i\beta\gamma$ subunit, which in turn causes the downstream activation of mitogen-activated protein kinases (MAPKs). $G_{ia}$ coupling also activates the cytosolic effector molecule phospholipase A2 (cPLA2), thus causing the cAMP-independent enrichment of Ca$^{2+}$ signalling and contraction. (Madamanchi A 2007) The $\beta$-ARs also have a negative feedback system involving receptor desensitization to avoid overstimulation. (Ferguson 2001) $\alpha$- and $\beta$-ARs stimulation induces an inotropic response by enhancing the phosphorylation of several regulatory proteins, such as MYBPC, cTnI, and PLB. MYBPC phosphorylation accelerates cross-bridge formation, and increase the contractile force. $\alpha$-ARs stimulation increases, whereas $\beta$-ARs activation reduces MYBPC phosphorylation. (Decker et al. 2010) During $\beta$-ARs stimulation, the Ca$^{2+}$ buffering remains constant because the stimulation causes two opposing effects on the buffering of [Ca$^{2+}$]; these two buffers effectively counterbalance each other. $\beta$-ARs stimulation increases the buffering through the phosphorylation of PLB, thus activates SERCA, while it also decreases the buffering by troponin phosphorylation. (Briston et al. 2014) PLB phosphorylation increases the SR Ca$^{2+}$ pumping, whereas cTnI phosphorylation increases the rate of Ca$^{2+}$ dissociation from myofilaments. $\beta$-ARs stimulation accelerates the cardiac relaxation, and is mediated entirely via the phosphorylation of PLB in the absence of an external mechanical load. (Li et al. 2000) PLB phosphorylation during $\beta$-ARs activation increases the cardiac contraction/shortening. (Wolska et al. 1996) In addition, $\beta$-ARs activation increases the rate of [Ca$^{2+}$], increases and the maximum level of [Ca$^{2+}$], because it increases the $I_{Ca}$ current and cause RyRs phosphorylations. (Yoshida et al. 1992; Wolska et al. 1996)
2.2 Stem cells

2.2.1 Stem cell characteristics

Stem cells are capable of performing either symmetrical or asymmetrical division. In symmetrical division, a stem cell divides into identical copies of the parent, and remains as a stem cell. In asymmetrical division, daughter cells do not remain as stem cells but differentiate into specific cell type. (Mountford 2008) The first element of life is a fertilized egg, which has the capacity to generate the whole organism. This capacity is known as totipotency, and this capability is maintained by the zygote up to the eight-cell morula stage. Blastocyst formation consists of outer trophoblast cells and undifferentiated inner cells called the inner cell mass (ICM). At this stage, inner cell mass are not totipotent, but they are pluripotent i.e., capable of differentiation into any cell type of an individual but not into extraembryonic tissues, such as the placenta and embryonic membranes. The main characteristic of embryonic stem cells is
pluripotency. Thus, they can yield any tissue type from all three dermal layers—endoderm, mesoderm and ectoderm. (Wobus & Boheler 2005) The first successful in vitro human embryonic stem cell (hESC) line was developed by Thomson and co-workers (Thomson et al. 1998). In Finland, hESCs are obtained from surplus embryos donated by couples undergoing in vitro fertilization (IVF) treatments with informed consent for research purposes. (Skottman 2010)

2.2.2 Induced pluripotent stem cell strategy

The breakthrough development of reprogramming fully differentiated cells back into a pluripotent stage occurred in 2006 (Takahashi & Yamanaka 2006) and revolutionized the conventional established theory of the one directional differentiation of cells. Yamanaka and co-workers used specific pluripotency factors such as octamer-binding transcriptional factor 3/4 (OCT3/4), sex determining region Y-box 2 (SOX2), Kruppel-like factor 4 (KLF4) and myelocytomatosis viral oncogene homolog (c-MYC), also known as Yamanaka factors to return fully differentiated mouse cells back to the pluripotent stage. Subsequently, in 2007, two different groups (Takahashi et al. 2007; Yu et al. 2007) reprogrammed human fibroblasts into pluripotent stage using a set of different factors, and revolutionized human stem cell research. Takahashi and co-workers (Takahashi et al. 2007) used the combination of retrovirally transfected transgenes (Yamanaka factors) to produce human induced pluripotent stem cell (hiPSC). Yu and co-workers (Yu et al. 2007) used lentivirally transfected factors of OCT4, SOX2, Nanog homeobox (NANOG) and lin-28 homolog A (LIN28).

These reprogramming techniques had some serious risks, such as the residual expression and re-activation of reprogramming factors, immunogenicity, the uncontrolled silencing of transgenes, and insertional mutagenesis. (Hu 2014) To surmount these hurdles, numerous methods utilizing different non-viral and non-integrative methods have been developed. Non-integrative methods involve viral (adeno and Sendai viruses) and non-viral approaches (plasmids, mRNA and protein). (Bayart & Cohen-Haguenauer 2013) (Figure 7)

Small molecule-mediated reprogramming methods have also been developed, which include chemicals and microRNAs (miRNAs) instead of transcriptional factors. These techniques decrease the risk of tumorigenesis because these methods eliminate the genetic modification of the host genome. (Ichida et al. 2009)
2.2.3 Differentiation into cardiomyocytes

During gastrulation, the three germ layers endoderm, ectoderm, and mesoderm are formed. Cardiomyocytes originate from the mesodermal germ layer. Cardiogenesis is the earliest process of organogenesis during embryonic development. (Rajala et al. 2011) The *in vitro* differentiation of cardiomyocytes mimics *in vivo* cardiac development. The cardiogenesis is complex process involving the following processes (1) mesoderm induction (2) patterning of mesoderm toward anterior mesoderm/ cardiogenic mesoderm (3) formation of cardiac mesoderm, and (4) maturation of cardiomyocytes. (Figure 8)
There are many methods for cardiac differentiation from pluripotent stem cells including (1) spontaneous embryoid body (EB) differentiation in suspension, (2) co-culture with mouse endoderm-like cells (END-2), and (3) guidance with defined growth factors in a monolayer or suspension. In suspension, cultured pluripotent stem cells differentiate into three dimensional cell aggregates called EBs. Itskovitz-Eldor and co-workers (Itskovitz-Eldor et al. 2000), and Kehat and co-workers (Kehat et al. 2001) successfully differentiated dhESCs into functional cardiomyocytes. Furthermore, Zhang and co-workers in 2009 (Zhang et al. 2009) were able to differentiate hiPSC-CMs. However, the efficiency of spontaneous cardiac differentiation with the EB method is less than 10% (Kehat et al. 2001). Another method for in vitro cardiac differentiation is co-culture with END-2 cells, which are derived from mouse P19 embryonal carcinoma cells. END-2 cells are treated with mitomycin C to prevent proliferation, and beating aggregates appear within 12-20 days using this method. (Mummery 2003) The precise function of END-2 cells in promoting cardiac differentiation remains unknown, but the removal of insulin and the secretion of prostaglandin I2 (PGI2) by END-2 cells are believed to impact the differentiation process (Xu et al. 2008).

The use of growth factors such as fibroblast growth factors (FGF), bone morphogenetic proteins (BPM), and Wnts is referred to as guided cardiac differentiation. In 2007, Laflamme and co-workers successfully differentiated hESCs into cardiomyocytes using Activin A and BMP4, and the differentiation efficiency was over 30% (Laflamme et al. 2007). Later, Zhang and co-
workers (Zhang et al. 2012) modified this differentiation technique using a matrix sandwich, in which cells were seeded on Matrigel™, and covered with Matrigel™. The addition of Activin A, BMP4, and basic FGF (bFGF) to the matrix improved the efficiency to 40-90%.

The reprogramming of fibroblasts into hiPSC using transcription factors triggers the idea that somatic cells can also be directly differentiated by a different set of transcription factors into functional cardiomyocytes, thus bypassing the first reprogramming into hiPSCs. During early heart development, Gata4, Mef2c, and Tbx5 are core transcriptional factors. The combination of these three developmental transcription factors can directly reprogram post-natal cardiac or dermal fibroblasts directly into functional cardiomyocytes (Ieda et al. 2010). In addition, a different set of transcriptional factors (Gata4, Mef2c, Tbx5, and Hand2) can reprogram adult mouse tail-tip and cardiac fibroblast directly into beating cardiomyocytes (Song et al. 2012).

2.3 Characterization of cardiomyocytes

2.3.1 Immunofluorescent staining

Structural characterization, including the sarcomeric proteins and myocyte morphology, can be studied by the immunofluorescent staining of cardiomyocytes. This immunofluorescence method provides information about the position of specific molecules, and the structure of cells. The backbone of this technique is the binding of antibodies to the antigens with high specificity and affinity. The fluorescent dye used to visualize a specific antigen can be conjugated to the primary antibody (direct), or a secondary antibody (indirect). These direct and indirect methods are not limited to immunofluorescence, but are also applicable to other techniques, such as flow cytometry, Western blotting, and immunohistochemistry. With the help of fluorescent microscope, the fluorescent dyes, which are also known as fluorophores or fluorochromes in immunofluorescence staining, allow the visualization of the target antigen. Technically, this method is relatively simple and straightforward, however, many variables must be recognized and optimized to generate a specific signal of interest. Briefly, samples are fixed to preserve cellular integrity, and blocking reagents are used to prevent non-specific antibody binding. Samples are then incubated with primary and secondary antibodies, and signal is visualized under a fluorescent microscope. (Donaldson 2015)

2.3.2 Real-time Polymerase chain reaction

The real-time polymerase chain reaction (RT-PCR) is also known as quantitative PCR. The key feature in this method is the DNA amplification is detected in real time using a fluorescent reporter. Because the number of gene copies increases during PCR, a corresponding increase in fluorescence occurs. Thus, the fluorescent reporter signal strength is directly proportional to the number of amplified deoxyribonucleic acid (DNA) molecules. (Kubista et al. 2006)
2.3.3 Western blotting

Western blotting is a technique for detecting proteins. This method involves sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the various proteins contained in a sample. Next, the separated proteins are transferred/blotted to a matrix, and stained with antibodies specific to a target protein. The bound antibodies attached to the target protein are preserved, and unbound antibodies are removed by washing. Then, the conjugated protein-antibody complex is visualized using radiation, colourimetric or chemiluminescence methods. The expression of target proteins in a given sample can be studied by analysing the location and intensity of the specific reaction, where the thickness of a band represents the amount of protein present. (Mahmood & Yang 2012)

2.3.4 Patch clamp

Recordings of APs and ionic currents from cardiomyocytes provide crucial information about their functional characteristics and aid in the study of cellular electrophysiology. The gold-standard patch-clamp technique allows the measurement of either $V_m$ or ions flowing across the membrane (Figure 9, Figure 10A) (Neher E 1976). A glass electrode with a sharp tip filled with a specific solution allows the bidirectional signal transformation between a cell and a complex electronic system. There are two modes in the patch clamp system: (1) current clamp (CC) and (2) voltage clamp (VC). In the CC approach, change in $V_m$ is measured while steady current is maintained. Thus, the AP i.e., change in $V_m$ is measured in CC mode. On the other hand, change in ion flux is measured in VC mode while maintaining constant $V_m$. Therefore, VC mode is used to study the quantity and kinetics of ionic currents. In the whole-cell patch clamp technique, the cell membrane beneath the tip of the glass electrode is ruptured to allow access to the interior of cell, and the solution inside the pipette enters the cell. (Sakmann & Neher 1984) In the perforated patch clamp technique, a perforating agent is used to create ion-permeable pores in the cell membrane that allow electrical access to the cells interior. There are various types of perforating agents available, including amphotericin-B, β-estin, and nystatin. These pores are permeable to small monovalent ions, such as $Na^+$, $K^+$, and $Cl^-$. However, these pores are impermeable to $Ca^{2+}$ and molecules such as ATP and glucose. The patch clamp technique is widely used in cardiovascular research using hiPSC-CMs to study the AP characteristics and arrhythmias, to identify and quantify cardiac ion channels, and for drug screening. (Liang et al. 2013; Lan, Andrew S. Lee, et al. 2013; Han et al. 2014)
2.3.5 Calcium imaging

Measurement of the $[\text{Ca}^{2+}]_i$ using $\text{Ca}^{2+}$ indicator dyes provides direct clues about the E-C characteristics of cardiomyocytes (Figure 10B). There is a wide range of $\text{Ca}^{2+}$ indicators with different excitation and emission spectra, $\text{Ca}^{2+}$ binding affinity, basal fluorescence, and cell permeability. Thus, $\text{Ca}^{2+}$ indicators should be carefully chosen in each experiment. A change in fluorescence intensity during measurement directly indicates the change in $[\text{Ca}^{2+}]_i$, concentration i.e., increased fluorescence intensity indicates higher $[\text{Ca}^{2+}]_i$, whereas decreased intensity indicates lower $[\text{Ca}^{2+}]_i$. The fluorescence intensity is also affected by the $\text{Ca}^{2+}$ indicator concentration, excitation path length, and local conditions such as viscosity and pH. However, photobleaching, indicator compartmentalization, or indicator extrusion can alter the fluorescence intensity. (Bootman et al. 2013; Fearnley et al. 2011) The measurement of $[\text{Ca}^{2+}]_i$ using fluorescence can be performed either by ratiometric or non-ratiometric methods. In the ratiometric method, the indicators exhibit an emission spectra shift or excitation spectra shift upon binding to $\text{Ca}^{2+}$, and the ratio between two fluorescence intensities is calculated. Thus, these indicators can be classified as dual emission or dual excitation. This method allows the correction of artefacts caused by bleaching, a change in focus, or a variation in laser intensity; however, it complicates data analysis more complicated. Examples of ratiometric indicators
are Fluo-2 and Indo-1. By contrast, non-ratiometric indicators show increased fluorescence intensity (intensity shift) upon Ca\(^{2+}\) binding. This intensity shift is often represented as F/F\(_0\) in case of normalization, where F represents the intensity of fluorescence emission and F\(_0\) is intensity at the start of an experiment. Non-ratiometric indicators include Fluo-3 and Fluo-4. (Bootman et al. 2013)

2.3.6 Microelectrode array

Microelectrode array (MEA) allows non-invasive and long-term electrophysiological recording using 32-252 electrodes underneath a cell cluster (Figure 10C). The MEA records the FD (defined as the time interval between the start of first sharp deflection to peak of second positive deflection) from the cell aggregates or sheet. (Reppel et al. 2004) Moreover, FP duration (FPD) prolongation is associated with QT prolongation, and there is linear relationship between FP and AP. (Halbach et al. 2003) With the help of MEA, not only FP, but also the conduction velocity, origin of excitation and direction of excitation spread can be studied. In addition, the first peak of FP waveform is closely related to the upstroke of AP, and the second FP peak nearly corresponds to the later repolarization of AP. (Asakura et al. 2015) This technique is less laborious and allow comparably high throughput compared to conventional patch clamp technique. Thus, MEA technique is widely used in drug screening for cardiotoxicity. (Zhu et al. 2017)

2.3.7 Video-based and cantilever-based movement analysis

Analysing the movement of cardiomyocytes during contraction and relaxation reveals the mechanical characteristics; such as beating pattern and contractile behaviour (Figure 10D). (Ahola et al. 2014; Maddah et al. 2015) This method is based on the video recording of beating cell, which is then analysed by different computational approaches. There is a linear relationship between FPD and contractile duration under normal conditions; this relationship between electrical and mechanical behaviour is the electro-mechanical (EM) coupling (Hayakawa et al. 2014). However, disease conditions e.g., LQT, can alter the EM ratio (Ter Bekke et al. 2015). The EM ratio is not only a useful indicator for many cardiovascular diseases, but also a marker of proarrhythmia (van der Linde et al. 2010). Furthermore, mechanobiological properties of cardiac cells such as contractile force, beating frequency and duration, and cellular elasticity are measured using atomic force microscopy (AFM). The mechanical behaviours are transduced by an AFM cantilever, which provides a robust biosensor platform. (Pesl et al. 2016) The AFM was used to measure the contractile force in DCM-specific hiPSC-CMs, which showed significantly weaker contraction forces compared to control hiPSC-CMs (Sun et al. 2012).
Figure 10. Functional testing of cardiomyocytes. (A) patch-clamp methods provide information about the cardiac membrane potential ($V_m$) (B) $Ca^{2+}$ imaging technique provides the information about intracellular $Ca^{2+}$ handling of cardiomyocytes (C) microelectrode array (MEA) provides the information about field potential and conduction velocity of cardiomyocytes (D) video-based technique provides the information about the mechanical behaviour of cardiomyocytes.

2.4 Hypertrophic cardiomyopathy

2.4.1 Genetic background

Although the heart can endure various pathological insults, sometimes the adaptive responses by the heart to maintain proper function fail; this can results in functional deficits or cardiomyopathies. Cardiomyopathies are diseases of the heart muscle associated with cardiac dysfunction. Cardiomyopathies can be inherited or acquired. Acquired cardiomyopathies are induced by hypertension, valvular diseases, diabetes, some chemotherapeutic agents, pregnancy, and alcohol consumption. Here we only focus on inherited cardiomyopathies. Mutation in > 900 genes expressed in cardiomyocytes causes a diverse range of cardiomyopathies. The inherited cardiomyopathies include DCM, restrictive cardiomyopathy (RCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC). In addition, many cardiomyopathies share clinical phenotypes. (Table 2) (Harvey & Leinwand 2011) In Europe, HCM was the most frequent cardiomyopathy, followed by DCM, and then ARVC and RCM (Charron et al. 2018).

Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disease. The true prevalence of HCM is unknown; however, it is estimated approximately 1 of 500 individuals between ages of 25 and 35 years have HCM. The prevalence of HCM is likely to be higher in older individuals because the penetrance of HCM is age dependent. (Semsarian et al. 2015) The pathological features of HCM are unexplained asymmetric or symmetric hypertrophy of the left ventricle, especially the septum, fibrosis and cardiomyocytes disarray of varying degree. HCM is the most common cause of premature SCD particularly in individuals younger than
35. HCM has variable penetrance, age of onset and distribution, and it exhibits large inter- and intra-familial heterogeneity. Some patients may exhibit severe limiting symptoms of dyspnoea, angina, syncope, progressive exercise intolerance, heart failure symptoms or unexpected SCD in worse cases, but some mutation carrier individuals do not present clinical markers or symptoms of disease. (Marsiglia & Pereira 2014; Maron & Maron 2013) HCM diagnosis is based on medical history (symptoms and family history), ECG and echocardiogram. Additional tests include, e.g., lab tests, exercise stress test, cardiac catheterization, CT scan, and magnetic resonance imaging. Genetic testing for HCM diagnosis is important in identification of affected or asymptomatic mutation carriers in families with known HCM-causing mutations. (Elliott et al. 2014)

The first mutation identified with linkage to HCM was in β-myosin heavy chain (MHC) gene. To date, more than 450 HCM-causing mutations in sarcomeric protein and myofilament-related genes have been identified. Mutations in β-MHC (30%), MYBPC (20%), cTnI (3-5%), and cTnT (3-5%) account for the majority of HCM. Furthermore, mutations in sarcomeric genes account for nearly 60% of all cases of HCM, and mutations in genes encoding sarcomere-associated proteins account for (%) of HCM; these proteins include myosin light chain kinase, muscle LIM protein, LIM binding domain 3, telethonin, vinculin, metavinculin, α-actinin 2, PLB, myogenin 2, and junctophilin-2. (Keren et al. 2008) Furthermore, mutations in same cardiac genes cause various types of cardiomyopathies. For example, mutations in cTnT (TNNT2) are commonly responsible for HCM, dilated DCM, RCM, and left ventricular noncompaction cardiomyopathy, which indicates a complex genotype-phenotype correlation. (Frey et al. 2012) Young children may present with left ventricle hypertrophy resembling HCM as part of other disease stages (eg., Noonan syndrome, mitochondrial myopathies, and metabolic disorders) unrelated to HCM-causing sarcomere protein mutations. (BJ 2002)

Although mutations in many cardiac genes associated with HCM have been identified, the molecular pathways involved in establishing phenotypes are unclear. The first proposed explanation was that the incorporation of mutant protein in sarcomere leads to a depressed contractile function, and triggers compensatory hypertrophy. However, these results were inconsistent in different studies. (Marsiglia & Pereira 2014)

### Table 2. HCM-causing mutations, their frequencies, and associated phenotypes (Keren et al. 2008)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Frequency</th>
<th>Associated phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myofilament genes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYH7</td>
<td>β-Myosin heavy chain</td>
<td>25–35%</td>
<td>Mild or severe HCM</td>
</tr>
<tr>
<td>MYBPC3</td>
<td>Myosin-binding protein C (cardiac type)</td>
<td>20–30%</td>
<td>Expression similar to MYH7, late onset</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Troponin T (cardiac muscle)</td>
<td>3–5%</td>
<td>Mild hypertrophy, sudden death</td>
</tr>
<tr>
<td>Gene</td>
<td>Protein Name</td>
<td>Frequency</td>
<td>Clinical Manifestation</td>
</tr>
<tr>
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<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>TNNI3</td>
<td>Troponin I (cardiac muscle)</td>
<td>&lt;5%</td>
<td>Extreme intrafamilial heterogeneity, no sudden death without severe disease</td>
</tr>
<tr>
<td>TPM1</td>
<td>Tropomyosin 1α</td>
<td>&lt;5%</td>
<td>Variable prognosis, sudden death</td>
</tr>
<tr>
<td>MYL2</td>
<td>Regulatory myosin light chain 2 (ventricular/cardiac-muscle isoform)</td>
<td>&lt;5%</td>
<td>Skeletal myopathy</td>
</tr>
<tr>
<td>MYL3</td>
<td>Essential myosin light chain 3</td>
<td>Rare</td>
<td>Skeletal myopathy</td>
</tr>
<tr>
<td>ACTC</td>
<td>α-Cardiac actin 1</td>
<td>Rare</td>
<td>Apical hypertrophy</td>
</tr>
<tr>
<td>TTN</td>
<td>Titin</td>
<td>Rare</td>
<td>Typical HCM</td>
</tr>
<tr>
<td>TNNC1</td>
<td>Troponin C, slow skeletal and cardiac muscles</td>
<td>Rare</td>
<td>Typical HCM</td>
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<td>α-Myosin heavy chain</td>
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<td>Late onset</td>
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<td>Myosin light chain kinase 2</td>
<td>Rare</td>
<td>Early onset</td>
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<td><strong>Z-Disc genes</strong></td>
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<tr>
<td>LDB3</td>
<td>LIM binding domain 3</td>
<td>Rare</td>
<td>Mainly sigmoidal HCM</td>
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<tr>
<td>TCAP</td>
<td>Telethonin</td>
<td>Rare</td>
<td>Typical HCM, variable penetrance</td>
</tr>
<tr>
<td>VCL</td>
<td>Vinculin/metavinculin</td>
<td>Rare</td>
<td>Obstructive midventricular hypertrophy</td>
</tr>
<tr>
<td>ACTN2</td>
<td>α-Actinin 2</td>
<td>Rare</td>
<td>Mainly sigmoidal HCM</td>
</tr>
<tr>
<td>MYOZ2</td>
<td>Myozenin 2</td>
<td>Rare</td>
<td>Typical HCM</td>
</tr>
<tr>
<td></td>
<td><strong>Calcium handling genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
<td>Rare</td>
<td>Typical HCM, variable penetrance</td>
</tr>
<tr>
<td>JPH2</td>
<td>Junctophilin 2</td>
<td>Rare</td>
<td>Typical HCM</td>
</tr>
</tbody>
</table>
2.4.2 HCM in the Finnish population

The Finnish population is genetically relatively homogenous with the presence of various HCM founder mutations, and Finland has a good public health care system. These factors offer unique possibilities to investigate genetic diseases in Finland. Mutations in the MHC gene are the most common genetic cause of HCM worldwide. However, this is not the predominant HCM-causing gene in the Finnish population; MHC mutations account for only 10% of familial and 3% of all HCM cases in Finland. (Jääskeläinen et al. 1998) By contrast, mutations in α-TPM causing HCM previously are not common worldwide, but one mutation (Asp175Asn) in α-TPM is more common in Finland (Jääskeläinen et al. 1998). In 2002, four novel variants (Gln1061X, IVS5-2A→C, IVS14-13G→A, and Ex25ΔLys) of MYBPC3 were identified HCM-causing mutations; among these mutations, Gln1061X was frequently found in Finland but is rare elsewhere (Jääskeläinen et al. 2002). All families with TPM1-Asp175Asn and MYBPC3-Gln1061X mutations were genealogically studied by tracing ancestors of 3-5 generations, but no consanguinity between families was recorded. (Jääskeläinen et al. 2004) The left ventricular end diastolic dimensions were increased in HCM patients with MYBPC3-Gln1061X compared with patients with TPM1-Asp175Asn. Thus, HCM patients with MYBPC3-Gln1061X may be more prone to cardiac dilation and cardiac failure. In addition, SCD because of cardiac failure or atrial fibrillation occurred in HCM patients with the MYBPC3-Gln1061X mutation. (Jääskeläinen et al. 2004) These first genetic studies were performed in one region of Finland and/or were based on a small patient population. Later, the prevalence of these two founder mutations was assessed in the nationwide Finnish patient population. The study showed that TPM1-Asp175Asn and MYBPC3-Gln1061X mutation causing HCM were found in 20 (6.5%) and 35 (11.4%) FinHCM patients respectively, and together account for ~18% of disease cases. (Jääskelainen et al. 2013) The most common HCM-causing mutations (TPM1-Asp175Asn and MYBPC3-Gln1061X) were also studied in family members. The overall penetrance of HCM-causing TPM1-Asp175Asn is 71% among relatives and 82% for all mutation carriers; the penetrance of MYBPC3-Gln1061X was 58% and 78% for relatives and carriers respectively. (Jääskelainen et al. 2013) Several SCDs in HCM patients carrying the TPM1-Asp175Asn mutation were reported, thus suggesting an intermediary prognosis (Jääskelainen et al. 2013). By contrast, HCM patients with the MYBPC mutation generally present milder clinical symptoms of disease, including a late onset of disease and lesser incidence of hypertrophy. (Niimura et al. 1998) HCM patients with TPM1-Asp175Asn have higher rate of family history of HCM (75% vs. 34%) and SCD (55% vs. 26%) compared with MYBPC3-Gln1061X HCM patients (Table 3). (Jääskelainen et al. 2013)

Table 3. Clinical characteristics of patients with HCM (Jääskelainen et al. 2013)

<table>
<thead>
<tr>
<th></th>
<th>All (n = 306)</th>
<th>TPM1-D175N (n = 20)</th>
<th>MYBPC3-Q1061X (n = 35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men/women</td>
<td>185/121 (60/40%)</td>
<td>9/11 (45/55%)</td>
<td>23/12 (66/34%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 ± 15 (16–85)</td>
<td>52 ± 15 (18–77)</td>
<td>49 ± 12 (21–76)</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
</tbody>
</table>

**Family history**

<table>
<thead>
<tr>
<th>HCM</th>
<th>83 (27%)</th>
<th>15 (75%)</th>
<th>12 (34%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sudden cardiac death</td>
<td>102 (33%)</td>
<td>11 (55%)</td>
<td>9 (26%)</td>
</tr>
</tbody>
</table>

**Cardiac symptoms**

<table>
<thead>
<tr>
<th>Dyspnea</th>
<th>131 (43%)</th>
<th>10 (50%)</th>
<th>11 (31%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chest pain</td>
<td>43 (14%)</td>
<td>4 (25%)</td>
<td>4 (11%)</td>
</tr>
<tr>
<td>Syncope/presyncope</td>
<td>84 (27%)</td>
<td>9 (45%)</td>
<td>9 (26%)</td>
</tr>
</tbody>
</table>

**History of arrhythmias**

<table>
<thead>
<tr>
<th>Paroxysmal atrial fibrillation</th>
<th>56 (18%)</th>
<th>4 (20%)</th>
<th>5 (14%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic atrial fibrillation</td>
<td>21 (7%)</td>
<td>4 (20%)</td>
<td>3 (9%)</td>
</tr>
<tr>
<td>Sustained ventricular tachycardia</td>
<td>10 (3%)</td>
<td>0 (0%)</td>
<td>2 (6%)</td>
</tr>
<tr>
<td>Ventricular fibrillation</td>
<td>14 (5%)</td>
<td>2 (10%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>History of systolic congestive heart failure</td>
<td>12 (4%)</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>History of ICD implantation</td>
<td>30 (10%)</td>
<td>2 (10%)</td>
<td>7 (20%)</td>
</tr>
<tr>
<td>History of myectomy</td>
<td>7 (2%)</td>
<td>2 (10%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

**Echocardiography**

<table>
<thead>
<tr>
<th>Maximal thickness of LV (mm)</th>
<th>20 ± 4 (10–38)</th>
<th>20 ± 2 (15–24)</th>
<th>22 ± 5 (15–38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDD (mm)</td>
<td>46 ± 7</td>
<td>43 ± 6</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>29 ± 7</td>
<td>27 ± 6</td>
<td>27 ± 6</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>68 ± 11</td>
<td>67 ± 11</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>Maximal flow velocity at LVOT (m/s)</td>
<td>1.8 ± 1.9</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>Bicycle ergometry (n = 214)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum load (W)</td>
<td>163 ± 69</td>
<td>120 ± 57</td>
<td>166 ± 65</td>
</tr>
<tr>
<td>Abnormal systolic BP response</td>
<td>21 (10%)</td>
<td>4 (20%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>Holter ECG registration (n = 249)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-sustained ventricular tachycardia</td>
<td>50 (20%)</td>
<td>2 (10%)</td>
<td>8 (23%)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>39 (16%)</td>
<td>4 (20%)</td>
<td>3 (9%)</td>
</tr>
</tbody>
</table>

Data are n (%) or mean ± SD. BP = blood pressure; HCM = hypertrophic cardiomyopathy; ICD = implantable cardioverter defibrillator; LV = left ventricle; LVEDD = left ventricular end-diastolic dimension; LVESD = left ventricular end-systolic dimension; LVOT = left ventricular outflow tract; MYBPC3 = cardiac myosin-binding protein C gene; TPM1 = alpha-tropomyosin gene.
Various modelling techniques such as biopsies, animal models, computational models, and hiPSC-CMs have been used for the better understanding of HCM. In an early study, septal myectomy was performed on a patient with obstructive HCM (48-y/o male). The results demonstrated the prolongation of APD (450 ms at 1 Hz), which was consistent with the prolonged QT/QTc interval recorded from the patient (476/455 ms). Additionally, a large late Na⁺ current and window current were recorded. (Barajas-Martinez et al. 2013) In another study, septal myectomy was performed on 26 HCM patients and APD prolongation, slower upstroke velocity, frequent occurrence of arrhythmias and abnormal Ca²⁺ handling were. (Coppini et al. 2013) However, because of ethical issues, the complex procedure and a limited supply of patients, there are only a few studies related to HCM using cardiac biopsy from patients.

Animals are also widely used to investigate cardiac physiology and diseases. The first genetically engineered animal model of HCM was with the Arg403Gln (R403Q) mutation of cardiac β-MHC gene in mice (Geisterfer-Lowrance et al. 1996). This model recapitulated the disease phenotype in several ways, including myocyte hypertrophy and disarray, interstitial fibrosis, and diastolic dysfunction. Similar disease phenotypes were found with a rabbit HCM model using the same R403Q mutation (Marian et al. 1999). Rabbit models have several advantages over mouse models; rabbits’ heart rate and ventricular sarcomere proteins are similar to those of humans. (Shephard & Semsarian 2009) A mouse model with truncated MYBPC (MBP-C¹⁰⁴⁺) produced the characteristics consistent with human HCM patients with the same mutation, such as a late onset of disease and significantly later display of histopathological features. (McConnell et al. 1999) HCM patients with mutations in cTnT generally exhibit mild or no ventricular hypertrophy, but they a have higher incidence of SCD. (Pasquale et al. 2012; Watkins et al. 1995) Mice expressing truncated cTnT have impaired cardiac contractility and relaxation with myocyte disarray, but they show no fibrosis or myocyte hypertrophy (Tardiff et al. 1998). HCM patients with mutated cTnI range from asymptomatic with a normal life to severe heart failure and even SCD. A mouse model with a mutation in cTnI presented myocytes disarray, interstitial fibrosis, and the upregulation of hypertrophic markers. (James et al. 2000) The α-TPM mutations in HCM are linked with variable ventricular hypertrophy, and a low incidence of SCD. The mouse model overexpressing α-TPM with a D175N mutation exhibited both systolic and diastolic function, myocyte disarray, hypertrophy, and fibrosis (Muthuchamy et al. 1999). Mutations in TPM are expressed in both myocardium and skeletal muscles. Thus, mutation in TPM in skeletal muscles showed higher sliding velocity and Ca²⁺ sensitivity (Kopylova et al. 2016).

Using the experimental data from human and animal models, computational models are also widely used for modelling the normal and diseased heart. A computational study constructing HCM AP model using experimental ionic currents, AP, and CaT recordings demonstrated a prolonged AP and CaT, diastolic Ca²⁺ overload, and decreased CaT amplitude (Passini et al. 2016). Recently hiPSC-CMs have been extensively used to study genetic cardiac diseases, and several articles related to HCM using hiPSC-CMs have been published (Lan, Andrew S. Lee, et al. 2013; Han et al. 2014; Wang et al. 2018; Li et al. 2018). Lan and co-workers were the first to produce hiPSC-CMs from HCM patients with a mutation (Arg663His) in MYH7 (Lan,
Andrew S. Lee, et al. 2013). They reported cellular enlargement, arrhythmias, and dysregulated Ca\(^{2+}\) cycling (Lan, Andrew S. Lee, et al. 2013). In 2014, another group produced hiPSC-CMs from HCM patients with a mutation (R442G) in MYH7, and showed disorganized sarcomeres and electrophysiological irregularities (Han et al. 2014). Similarly, hiPSC-CMs obtained from HCM patients with a mutation (c.1358-1359insC) in MYBPC3 exhibited ~50% lower MYBPC3 mRNA and MYBPC protein levels compared with control cells, larger cell size, and altered gene expression (Prondzynski et al. 2017). Recently, HCM-specific hiPSC-CMs with a TnT-179N mutation displayed sarcomere disorganization, increased systolic function, myofilament Ca\(^{2+}\) sensitivity, and impaired relaxation (Wang et al. 2018). Furthermore, the modelling of HCM using hiPSC-CMs carrying an m.2336T>C mutation in the mitochondrial 16S rRNA gene (MT-RNR2) showed mitochondrial dysfunction, reduced levels of mitochondrial proteins, elevated [Ca\(^{2+}\)], and electrophysiological abnormalities (Li et al. 2018).
3. Aims of the study

The aims of the present thesis are as follow:

1. Establish *in vitro* HCM-specific models using hiPSC-CMs (Study I);
2. Investigate the effect of adrenaline, and examine the efficacy of bisoprolol as an antiarrhythmic drug in HCM-specific hiPSC-CMs (Study II);
3. Understand the interrelation between $V_m$ and CaT in hiPSC-CMs (Study III).

The specific aims of each study were as follows:

The aims of study I were to establish the *in vitro* cell models using patient-specific hiPSC-CMs from Finnish HCM patients carrying *TPM1-Asp175Asn* and *MYBPC3-Gln1061X*, and to evaluate and compare these models with each other and with control hiPSC-CMs,

The aims of study II were to examine the effects of different concentrations of adrenaline in HCM-specific hiPSC-CMs both during exposure and after wash-out of adrenaline, and to evaluate the efficacy of bisoprolol as an antiarrhythmic agent in HCM.

The aim of study III was to understand the interrelation between $V_m$ and CaT in hiPSC-CMs using a conventional patch-clamp technique and calcium imaging system.
4. Material and Methods

4.1 Ethical consideration

Our group (Heart group, Biomeditech, UTA) has permission from the Ethics Committee of Pirkanmaa Hospital District to conduct this research on hiPSC lines (Aalto-Setälä R08070). Patients donating skin biopsies provided written consent, and received both written and oral information about the study in the Heart Hospital, Tampere University Hospital. (Study I-III)

4.2 Generation and characterization of patient-specific hiPSCs (I-III)

hiPSC lines were generated from skin’s fibroblasts either with the Sendai reprogramming OCT4, KLF4, c-MYC, and SOX2 vectors using the CytoTune-iPS Reprogramming Kit (Life Technologies Ltd., Paisley, UK) or using the pMX retroviral OCT4, KLF4, c-MYC, and SOX2 vectors with or without LoxP sites. UTA.13602.HCMT, UTA.02912.HCMT, and UTA.04511.WT hiPSC lines were generated using Sendai vectors, UTA.07801.HCMM and UTA.06108.HCMM were generated using pMX retroviral vectors with Cre-LoxP site and UTA.04602.WT was generated by using pMX retroviral vectors without LoxP sites. The hiPSC lines were cultured on mouse embryonic fibroblast (MEF) feeder cell layers (26000 cells/cm², CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany) in culture medium consisting of knockout-DMEM (ko-DMEM, Gibco, Life Technologies Ltd.) supplemented with 20% knockout serum replacement (ko-SR, Gibco, Life Technologies Ltd.), 1% nonessential amino acids (NEAA, Lonza Group Ltd., Basel, Switzerland), 2 mM GlutaMax (Gibco, Life Technologies Ltd.), 50 U/mL penicillin/streptomycin (Lonza Group Ltd.), 0.1 mM 2-mercaptoethanol (Gibco, Life Technologies Ltd.), and 4 ng/mL basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ, USA). (Study I-III)

DNA samples from the hiPSC lines were prepared with TaqMan Sample-to-SNP Kit (Applied Biosystems, Life Technologies Ltd.) and the presence of MYBPC3-Gln1061X and TPM1-Asp175Asn mutations in the patient-specific hiPSC lines were confirmed using custom TaqMan SNP genotyping assays (Applied Biosystems, Life Technologies Ltd.) RNA samples were collected and extracted from hiPSC-derived cardiomyocytes (UTA.04511.WT, UTA.02912/3.HCMT, UTA.07801.HCMM, and UTA.06108.HCMM) with Norgen’s Total RNA Purification Plus Kit (Norgen Biotek Corp., Thorold, Canada). The mRNA expression of TPM1-Asp175Asn or MYBPC3-Gln1061X mutation in the hiPSC-derived CMs was studied using custom TaqMan SNP genotyping assays (Thermo Fisher Scientific, Massachusetts, USA). The presence of endogenous pluripotency genes Nanog, SOX2, REX1, OCT4, and c-MYC and the absence of the virally imported exogenes (OCT4, SOX2, c-MYC, and KLF4) were confirmed by RT-PCR. GAPDH was used as an endogenous control. The hiPSC colonies were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA), and stained with primary antibodies against Nanog (R&D systems Inc.,Mimmesota,USA), OCT4 (R&D
systems Inc.), SOX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRA-1-60 (Millipore, Billerica, MA, USA), and TRA-1-81 (Millipore), and appropriate with secondary antibodies. Vectashield (Vector Laboratories Inc., Burlingame, USA) containing 4’,6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. The karyotypes of hiPSC lines were analysed by G-banding (Medix Laboratories, Espoo, Finland) or by the KaryoLite assay (Turku Centre for Biotechnology, University of Turku, Turku, Finland). The pluripotency of hiPSC lines was confirmed by embryoid body (EB) formation. EBs were cultured in EB medium consisting of KO-DMEM with 20% fetal bovine serum (FBS, Biosera, Boussens, France), 1% NEAA (Lonza Group Ltd.), 2 mM GlutaMax (ThermoFisher Scientific), and 50 U/mL penicillin/streptomycin (Lonza Group Ltd) for 4 – 6 weeks. The presence of all three germ layers - endoderm (AFP, SOX17), ectoderm (SOX1, NESTIN, and Musashi), and mesoderm (KDR, alpha cardiac actin), was analysed by RT-PCR. (Study I)

Table 4. Characteristics of individuals included in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mutation</th>
<th>Sex</th>
<th>Age</th>
<th>IVS (mm)</th>
<th>Other symptoms</th>
<th>SCD in family</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTA.04602.WT</td>
<td></td>
<td>F</td>
<td>56</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTA.04511.WT</td>
<td></td>
<td>M</td>
<td>34</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTA.13602.HCMT</td>
<td>TPM1-Asp175Asn</td>
<td>F</td>
<td>48</td>
<td>16</td>
<td>Collapsed when 20 years old (normal heart structure)</td>
<td>mother at age 51</td>
<td>none</td>
</tr>
<tr>
<td>UTA.02912.HCMT</td>
<td>TPM1-Asp175Asn</td>
<td>M</td>
<td>33</td>
<td>26</td>
<td>Asymptomatic</td>
<td>One family member at age 21</td>
<td>β-blocker</td>
</tr>
<tr>
<td>UTA.02913.HCMT</td>
<td>TPM1-Asp175Asn</td>
<td>M</td>
<td>33</td>
<td>26</td>
<td>Asymptomatic</td>
<td>One family member at age 21</td>
<td>β-blocker</td>
</tr>
<tr>
<td>UTA.06108.HCMM</td>
<td>MYBPC3-Gln1061X</td>
<td>M</td>
<td>55</td>
<td>22</td>
<td>Asymptomatic</td>
<td>father at age 36 uncle at age 38</td>
<td>none</td>
</tr>
<tr>
<td>UTA.07801.HCMM</td>
<td>MYBPC3-Gln1061X</td>
<td>M</td>
<td>61</td>
<td>25</td>
<td>Atrial fibrillation</td>
<td>none</td>
<td>β-blocker ICD</td>
</tr>
</tbody>
</table>

IVS: Intraventricular septum, SCD: Sudden cardiac death, ICD: Implantable cardioverter defibrillator

4.3 Differentiation and characterization of hiPSC-CMs

hiPSCs were differentiated into cardiomyocytes by coculturing with mouse visceral endodermal-like cells (END-2) (Mummery 2003). The beating areas were excised from cocultures and dissociated into single cells in EB medium by Collagenase A (Roche Diagnostics, Mannheim, Germany) and plated on 0.1% gelatin-coated cover slips/well plates. (Mummery 2003) (Study I-III)

Dissociated cardiomyocytes were fixed with 4% paraformaldehyde (Sigma-Aldrich) and stained with cTnT (1:2000, Abcam, Cambridge, UK), MYBPC (1:400, Santa Cruz
Biotechnology), and TPM1 (1:200, Santa Cruz Biotechnology) primary antibodies. The next day, cardiomyocytes were labelled with secondary antibodies. (Study I) Images were obtained with Olympus IX51 phase contrast microscope equipped with fluorescence optics and Olympus DP308W camera (Olympus Corporation, Tokyo, Japan) or with Zeiss AxioScope A1 fluorescent microscope and Zeiss AxioCam MRc5 camera (Carl Zeiss, Jena, Germany). Similarly, for study III fixed cardiomyocytes were stained with cTnT (1:500; Abcam), I_{Ca,L} (1:500; Alomone labs, Jerusalem, Israel) primary antibodies, followed by labelling with secondary antibodies. Vectashield (Vector Laboratories Inc., Burlingame, USA) containing DAPI was used for nuclear staining. The z-stack images were captured with Nikon A1 confocal laser-scanning microscope (Nikon, Tokyo, Japan) using 60x oil immersion objectives (N.A.=1.4). The images were processed using Image J (National Institute of Health, Maryland, USA) and photoshop (Adobe Photoshop, San Jose, USA).

4.5 Patch clamp, calcium imaging, and their synchronized measurement

In studies I-III, APs were recorded in gap-free mode using a conventional patch clamp in perforated patch configuration using amphotericin B at a final concentration of 0.24 mg/mL (Studies I-III). Data acquisition was conducted using an Axon Series 200B patch-clamp amplifier connected to a Digidata 1440a AD/DA converter driven by pCLAMP 10.2 software (all from Molecular devices, LLC). Current-clamp recordings were digitally sampled at 20 kHz and filtered at 2 kHz using a low-pass Bessel filter on the recording amplifier. The coverslips containing dissociated hiPSC-CMs were transferred to a RC-24N recording chamber (Warner Instruments, Hamden, USA) and mounted on an inverted Olympus IX71 microscope (Olympus Corporation). The CMs were continuously perfused with extracellular solution preheated to 35-36°C by an SH-27B inline heater controlled by a TC-324B unit (all from Warner Instruments Inc., Hamden, USA). The extracellular solution contained 143 mM NaCl, 4.8 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH was adjusted to 7.4 with NaOH). Patch pipettes (Harvard Apparatus Ltd., Holliston, USA) were polished with a MF-830 microforge (Narishige Int., Tokyo, Japan). The patch electrodes had a tip resistance of 3.0–3.5 MΩ when filled with intracellular solution containing 132 mM KMeSO₄, 20 mM KCl, 4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N''-tetraacetic acid (EGTA), 1 mM MgCl₂, and 1 mM CaCl₂ (pH adjusted to 7.2 with KOH).

In study I, [Ca²⁺], was measured from hiPSC-derived CMs, using 4 μM Fura-2 AM (Thermo Fisher Scientific), which was loaded for 30 minutes in extracellular medium consisting 137 mM NaCl, 5 mM KCl, 0.44 mM KH₂PO₄, 20 mM HEPES, 4.2 mM NaHCO₃, 5 mM D-glucose, 2 mM CaCl₂, 1.2 mM MgCl₂, and 1 mM Na-pyruvate dissolved in H₂O (pH adjusted to 7.4 with NaOH). Coverslips containing the hiPSC-CMs were mounted in an RC-25 recording chamber and continuously perfused with extracellular solution preheated to 35-36°C by an SH-27B inline heater controlled by a TC-324B unit (all from Warner Instruments Inc.). The spontaneously beating CMs were imaged with an inverted IX70 microscope using a UApo/340
x20 air objective (Olympus Corporation) and an ANDOR iXon 885 CCD camera (Andor Technology, Belfast, Northern Ireland) synchronized with a Polychrome V light source by a real time DPS control unit. TILLvisION or Live Acquisition software (TILL Photonics, Munich, Germany) was used for recording. Fura-2 AM was excited with 340 nm and 380 nm light, and emission was recorded for 10–30 seconds at 505 nm.

In study III, the patch clamp system and Ca^{2+} imaging system were performed synchronously, which was achieved with the synchronization pulse sent from the imaging system to the patch clamp system when Ca^{2+} imaging started; the synchronization pulse stopped once the Ca^{2+} imaging system stopped recording. The extracellular solution and pipette solutions were similar as those in study I for APs recording. In addition, we used 4 µM Fluo-4 AM (Thermo Fisher Scientific), which was excited at 490nm and emission was recorded through an Olympus U-MF2 Alexa 488 band-pass filter cube (excitation 470-495, emission 525/500 nm). The sampling interval was 20 ms.

4.6 Data analysis

In study I, regions of interest (ROIs) and background noise were selected to extract CaT. Data are presented as the ratios of 340/380 nm (F340/F380).

In studies I-III, recorded APs were analysed with home-made OriginTM 9.1 (OriginLab Corp., Northampton, USA) scripts to extract the following parameters: beats per minute (BPM), AP duration at 50% and 90% repolarization (APD50 and APD90), AP amplitude (APA), upstroke velocity (dV/dt) and maximum diastolic potential (MDP). The hiPSC-CMs were categorized as ventricular-like when they showed APD90/APD50 < 1.3 and APA > 90 mV, and they were categorized as atrial-like CMs when they showed APD90/APD50 > 1.35. Similarly hiPSC-CMs were categorized as nodal-like cardiomyocytes when they showed APD90/APD50 > 1.3 and APA < 80 mV with slower a dV/dt.

In study II, beat-rate-variabilities (BRVs) were assessed from a minimum of 30 consecutive APs without the presence of arrhythmias. SD1 is the standard deviation (SD) of the instantaneous beat-to-beat interval variability. SD2 is the SD of the long-term interval variability (LTV). SD1 and SD2 were calculated using the formulas SD1^2 = 1/2*SDSD^2 and SD2^2 = 2SDRR^2-1/2*SDSD^2 respectively; SDRR represents the standard deviation of RR intervals and SDSD represents the standard deviation of differences between adjacent RR intervals. The beat-to-beat variability at 50% and 90% repolarization (STV-50 and STV-90) were assessed in the same APs selected for BRV using the formula STV-50 = \[ \sum |APD50n+1-APD50n|/n\sqrt{2} \] and STV-90 = \[ \sum |APD90n+1-APD90n|/n\sqrt{2} \] respectively. N represents the number of APs used to calculate variabilities. These values were calculated using custom-made software in Matlab R2015a (The MathWorks, Inc., Natick, USA).
For study III, CaTs were normalized as $\Delta F/F_0$ using LiveAcquisition software. The CaTs were analysed with Clampfit software to extract CaT at 50% and 90% of repolarization (CaT50 and CaT90), and time-to-peak (time taken for CaT to reach its peak intensity).
5. Results

5.1 Generation of patient-specific hiPSCs and hiPSC-CMs

In these studies, hiPSC lines were obtained from six individuals. Detailed information for these individuals is included in Table 4. The hiPSCs lines were derived from healthy individuals (Studies I-III) and HCM patients (Studies I-II). The iPSC lines formed colonies expressing pluripotency proteins Nanog, OCT3/4, SOX2, TRA-1-60, and TRA-1-81 (Figure 11A). Virally transferred exogenous genes were silenced, and endogenous pluripotent genes were turned on (Figure 11B). Karyotypes of the hiPSC lines were normal (Figure 11C). The presence of TPM1-Asp175Asn or MYBPC3-Gln1061X mutation in the hiPSC lines was confirmed using custom TaqMan SNP Genotyping Assays (Study I). All hiPSC lines were differentiated into cardiomyocytes by co-culturing with END-2 cells, and beating aggregates were formed after 14-20 days (Studies I-III).

Figure 11. Characterization of the hiPSC lines. (A) hiPSCs formed colonies expressing Nanog, OCT4, SOX2, TRA-1-60, and TRA-1-81. Scale bars: 200 μm. (B) The virally transferred Sendai exogenes were silenced in hiPSCs. + indicates positive controls, for which RNA was extracted from cells 1 week after transduction. hiPSCs expressed endogenous was used as a housekeeping control. (C) The hiPSC line was karyotypically normal (D) The pluripotency of hiPSCs was confirmed by in vivo teratoma assays; all three germ layers (mesoderm, endoderm, and ectoderm) were formed.
5.2 Modelling HCM using hiPSC-CMs

5.2.1 Characteristics of hiPSC-CMs
hiPSC-CMs obtained from healthy individuals and HCM patients expressed cTnT, MYBPC and TPM proteins (Figure 12A). The levels of the cTnT, MYBPC, and TPM proteins were evaluated by Western blot analysis with both control and HCM-specific hiPSC-CMs (Figure 12B). The expression of the sarcomeric genes MYBPC3, TNNT2, ACTN2, TTN, MYL7, and MYL9 was significantly higher in both HCMT-CMs and HCMM-CMs than in WT-CMs (P < 0.005 in all cases, Figure 12C). The expression of TPM1 and TNNC1 was significantly increased only in the HCMM when compared to WT (P < 0.005 in both cases, Figure 12C). Importantly, truncated MYBPC protein was not observed in hiPSC-derived cardiomyocytes with the MYBPC3-Gln1061X mutation (Data not shown). (Study I)

Figure 12. Expression of sarcomeric proteins and genes in hiPSC-CMs. (A) Representative images of WT-CMs, HCMT-CMs, and HCMM-CMs stained with antibodies for cTnT (red) and MYBPC (green) proteins. Scale bars are 100 μm. (B) All cell lines expressed MYBPC, cTnT, and TPM proteins. (C) Gene expression profiles in WT-
CMs, HCMT-CMs, and HCMM-CMs. * or $ P<0.05$ Mann-Whitney $U$ test with Bonferroni’s correction; * represents HCMT or HCMM versus WT, and $ represents HCMT versus HCMM.

5.2.2 Baseline properties of hiPSC-CMs

In study I, the AP recordings from spontaneous beating hiPSC-CMs showed that both HCMT-CMs and HCMM-CMs had significantly longer APD90 than WT-CMs, and that the APD90 of HCMT-CMs was also significantly longer than that of HCMM-CMs ($ P<0.05$; WT-CMs: 323.6±13.9 ms, HCMT-CMs: 433.1±14.0 ms, HCMM-CMs: 377.6±15.0 ms). By contrast, beating rates were significantly lower in HCMT-CMs and HCMM-CMs than in WT-CMs ($ P<0.05$; WT-CMs: 58.1±2.3 ms, HCMT-CMs: 48.4±1.5 ms, HCMM-CMs: 47.1±1.8 ms). However, no significant differences were found for the MDP among these groups. To understand the remodelling of ion channels in HCM, we recorded the $I_{\text{Ca}}$, $I_{\text{to}}$, and $I_{\text{K1}}$ current densities from WT-CMs, HCMT-CMs, and HCMM-CMs in study II. The results demonstrated that the $I_{\text{Ca}}$ current densities were significantly higher in both HCMT-CMs and HCMM-CMs compared with WT-CMs ($ P<0.05$; WT-CMs versus HCMT-CMs from −10 mV to 70 mV, and WT-CMs versus HCMM-CMs from 10 mV to 60 mV; Figure 13A). However, no significant difference was found between HCMT-CMs and HCMM-CMs. By contrast, the $I_{\text{to}}$ current densities were significantly lower in both HCMT-CMs and HCMM-CMs compared to WT-CMs ($ P<0.05$, WT-CMs versus HCMT-CMs and HCMM-CMs from 20 mV to 70 mV; Figure 13B). No significant differences in $I_{\text{to}}$ current densities were found between HCMT-CMs and HCMM-CMs at any potential. The $I_{\text{K1}}$ current densities were not significantly different at any test potentials among groups (Figure 13C). Furthermore, beat rate variabilities (SD1 and SD2) and APD90 variability were measured from hiPSC-CMs. The results showed that SD1 and SD2 were significantly higher in both HCMT-CMs and HCMM-CMs than in WT-CMs (Figure 13D-E). However, STV-APD90 was significantly higher only in HCMT-CMs compared to WT-CMs (Figure 13F). (Study II)
5.2.3 Classification of arrhythmias in hiPSC-CMs

In study I, measurement of $[\text{Ca}^{2+}]$ using Fura-2 AM demonstrated four types of $\text{Ca}^{2+}$ abnormalities: (1) low/middle peaks (small amplitude events at the end of the CaT; Figure 14A), (2) double peaks (two/three peaks that do not return to baseline; Figure 14B), (3) oscillation (more than three peaks that do not return to baseline; Figure 14C), and (4) plateau (CaT with prolonged decay time). Furthermore, HCMT-CMs had significantly more $\text{Ca}^{2+}$ abnormalities than WT-CMs and HCMM-CMs ($P < 0.05$). In APs recording, delayed afterdepolarization (DAD) and early afterdepolarization (EAD) were recorded from hiPSC-CMs, and HCM-specific hiPSC-CMs exhibited higher percentage of both types arrhythmias (Studies I and II). The most common type of arrhythmia recorded from hiPSC-CMs was DAD, which was defined as the presence of low-amplitude abnormal depolarization after successive APs (Figure 14E). Under baseline conditions, the DAD rate was significantly higher in HCMM-CMs than in WT-CMs (study I). Moreover, in study II, EAD events were further classified into four subgroups: (1) phase 3 EAD (defined as the initiation of the next AP during the third phase of repolarization; Figure 14F), (2) burst (defined as the sudden increase in beating rate triggered by phase 3 EAD; Figure 14G), (3) quasi-equilibrium state EAD (QES-EAD, defined as a sudden decrease in APA leading to temporary non-beating $V_m$ or oscillation and self-recovery to spontaneous APs; Figure 14H), and (4) VT (defined as phase 3 EAD mediating
triggered activity followed by an increased beating rate (Figure 1 I-K). Notably, in phase 3 EAD, the triggered AP had distinct characteristics; some triggered APs had smaller APA and APD than APs constituting phase 3 EAD, whereas some triggered AP had longer APD than AP constituting phase 3 EAD. A distinct characteristic observed in some of the bursts was an increase in the upstroke velocity in the AP comprising the burst EAD. The duration and beat rate of the burst EAD ranged from 0.3 s to 27 s and from 50 BPM to 260 BPM, respectively. Similarly, the take-off potential for burst in our study ranged from −70 mV to −30 mV. Furthermore, an increase in upstroke velocity in APs comprising QES-EAD events was observed in some cases. The take-off potential and duration of QES-EAD recorded in our study varied from −55 mV to −6.5 mV and from 1.4 s to 22.3 s, respectively. The main characteristics of VT-EAD were a steady APD and MDP during triggered activity. Our results showed that the beat rate and MDP of VT ranged from 70 BPM to 254 BPM and from −69 mV to −45 mV, respectively. Furthermore, another distinguished feature of VT was the possibility of progressively extended triggered activity. Therefore, VTs were again subdivided on the basis of the duration of triggered activity into non-sustained (NSVT, duration <30 s, self-terminated; Figure 1 I), sustained (SVT, duration >30 s, self-terminated; Figure 1 J) and non-recovered (NRVT, not recovered; Figure 1 K).

These arrhythmias were present either exclusively or in the presence of another type of arrhythmia in hiPSC-CMs. The first four types of arrhythmias (DAD, phase 3 EAD, burst and QES-EAD) were observed in WT-CMs, HCMT-CMs and HCMM-CMs. However, the VT-type of arrhythmias were only observed in HCMT-CMs. To confirm that VT-type of arrhythmias were exclusively present in HCMT-CMs, we decided to include the clonal line UTA.02912.HCMT with the TPM1-Asp175Asn mutation, which is designated UTA.02913.HCMT (Table 4). In APs recording, we were also able to record VTs in hiPSC-CMs derived from the UTA.02913.HCMT line and therefore confirmed that VTs were present in HCMT-CMs.
Figure 14. Classification of the arrhythmias observed in hiPSC-CMs. Types of abnormal Ca$^{2+}$ handling recorded from Ca$^{2+}$ imaging: (A) low/middle peaks, (B) double peaks, (C) oscillation, and (D) plateau. Types of arrhythmias observed in current clamp recording (E) delayed after depolarization, (F) phase 3 early after depolarization (EAD), (G) burst, (H) severe, (I) non sustained ventricular tachycardia (VT), (J) non-returned VT, and (K) sustained VT.
5.2.4 Effect of adrenaline on the occurrence of arrhythmias

To determine whether adrenaline causes any adverse effects, one concentrations of adrenaline at a time (0.1 nM, 0.5 nM, 1 nM, 10nM, 100nM, 1µM, and 10 µM) was applied to hiPSC-CMs in study II. After the administration of adrenaline, the spontaneous beat rates were significantly increased ($P<0.05$, paired $t$-test), but the APD90s were significantly decreased ($P<0.05$, paired $t$-test) from pre-adrenaline values in a concentration-dependent manner in WT-CMs, HCMT-CMs, and HCMM-CMs. Similarly, dV/dT was not significantly changed by any concentration of adrenaline in WT-CMs, HCMT-CMs, or HCMM-CMs. Furthermore, no significant differences of SD1 or SD2 were observed among WT-CMs, HCMT-CMs, and HCMM-CMs. However, the percentage change in APD90 was significantly lower at 1 nM adrenaline for HCMM-CMs ($P<0.05$), and at 10 nM adrenaline for both WT-CMs and HCMT-CMs (*, # $P<0.05$). We quantified various types of arrhythmias before exposure to adrenaline, during the administration of adrenaline, and immediately after washing out the adrenaline. No significant differences in the DAD rate were found for any adrenaline concentration tested in comparison before, during, and immediately after wash-out for WT-CMs, HCMT-CMs, and HCMM-CMs (Table 5). The phase-3 EAD rate was quantified as the number of phase 3 EAD events over a certain period of time (number of phase 3 EAD events/ total time period). Similarly, the phase-3 EAD rate was not significantly changed at any concentration tested for all three groups (Table 6). In the presence and immediately after the wash-out of adrenaline ($\geq 100$ nM), no occurrence of burst was observed in WT-CMs (Table 7). The frequency of burst was calculated as the total number of burst events/total number of cells. Furthermore, bursts were observed during and immediately after wash-out at all concentrations of adrenaline tested for HCMT-CMs. Notably, during the administration of 0.5 nM adrenaline, the frequency of burst was the highest (38%) among the concentrations of adrenaline tested in HCMT-CMs. However, the percentage of burst events was the highest after the wash-out of 1 nM adrenaline in HCMT-CMs. Moreover, the percentages of burst events were similar during the administration of 0.1 nM, 0.5 nM and 1 nM adrenaline; however no burst was recorded during the administration of $\geq 10$ nM adrenaline. In addition, the percentage of burst events was the highest after the wash-out of 1 nM adrenaline. The frequency of QES-EAD events was calculated as the total number of QES-EAD events/total number of cells (Table 8). During the administration of 0.1 nM and 1nM adrenaline, the frequencies of QES-EAD events were higher (14% and 13%) than at other concentrations of adrenaline tested in HCMT-CMs. Similarly, QES-EAD events were observed in similar percentages immediately after the wash-out of 1 nM, 1µM, and 10 µM adrenaline in HCMT-CMs. In HCMM-CMs, the frequency of QES-EAD events was the highest during the administration of 0.1 nM adrenaline (21%), and immediately after the wash-out of 1 nM adrenaline (13%). Only NSVT was observed during the baseline recording of APs from HCMT-CMs (Table 9). During the administration of 0.1 nM, 0.5 nM, 1 nM and 10 nM adrenaline, NSVT and SVT were observed; however, these arrhythmias were not observed during the administration of $\geq 100$ nM adrenaline. In addition, NRVT was observed only immediately after the wash-out of 10 nM adrenaline in HCMT-CMs. Furthermore, SVT and NRVT were also observed immediately after the wash-out of 0.5 nM, 1nM, 10 nM, 100 nM, and 1 µM adrenaline.
Table 5. DAD rate (1/min) in hiPSC-CMs. The frequency of DAD events was calculated as the total number of DADs/total number of APs.

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<th>Washout</th>
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The DAD rates did not change significantly from baseline during or immediately after the administration of different concentrations of adrenaline in WT-CMs, HCMT-CMs and HCMM-CMs. (ns: Friedman, posthoc Dunn test). * or # P<0.05, **P<0.005 (WT-CMs vs HCMT-CMs or HCMM-CMs; Kruskal Wallis, post hoc Dunn test). Data are presented as the mean±SEM. n represents the number of cells used in each experiment.
Table 6. Phase 3 EAD rate (1/10min) in hiPSC-CMs. The frequency of phase 3 EAD rate was calculated as the total number of Phase 3 EADs/total time.

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The phase 3 EAD rate did not change significantly from baseline during or immediately after the administration of different concentrations of adrenaline in WT-CMs, HCMT-CMs and HCMM-CMs. (ns: Friedman, post hoc Dunn test). * or # P<0.05 (WT-CMs vs HCMT-CMs or HCMM-CMs; Kruskal Wallis, post hoc Dunn test). Data are presented as the mean±SEM. n represents the number of cells used in each experiment.
Table 7. Categorical analysis of burst events in hiPSC-CMs. The frequency of burst was calculated as the total number of bursts/total number of cells.

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<td>26% (8)</td>
<td>13% (4)</td>
<td>6% (2)</td>
<td></td>
<td>18</td>
<td>17% (3)</td>
<td>0% (0)</td>
<td>6% (1)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>12</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>19</td>
<td>5% (1)</td>
<td>5% (1)</td>
<td>21% (4)</td>
<td></td>
<td>11</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td></td>
<td>11</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>24</td>
<td>0% (0)</td>
<td>4% (1)</td>
<td>13% (3)</td>
<td></td>
<td>10</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td></td>
</tr>
<tr>
<td>10000</td>
<td></td>
<td>14</td>
<td>7% (1)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>29</td>
<td>7% (2)</td>
<td>7% (2)</td>
<td>3% (1)</td>
<td></td>
<td>14</td>
<td>14% (2)</td>
<td>0% (0)</td>
<td>7% (1)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as percentages. The percentages were rounded to whole number. n represents the number of cells used in each experiment. The value in parenthesis represents the number of bursts that occurred in each experiment.
Table 8. Occurrence of QES-EAD in hiPSC-CMs. The frequency of QES-EAD was calculated as the total number of QES-EAD events/total number of cells.

<table>
<thead>
<tr>
<th>nM</th>
<th>WT-CMs</th>
<th>HCMT-CMs</th>
<th>HCMM-CMs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>0.1</td>
<td>13</td>
<td>0% (0)</td>
<td>8% (1)</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>8% (1)</td>
<td>8% (1)</td>
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<tr>
<td>1000</td>
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<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>10000</td>
<td>14</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
</tbody>
</table>

Data are presented as percentages. The percentages were rounded to whole numbers. n represents the number of cells used in each experiment. The value in parenthesis represents the number of QES-EAD arrhythmia events.
### Table 9. Occurrence of VTs in HCMT-CMs. Three types of VTs were observed in HCMT-CMs.

<table>
<thead>
<tr>
<th>nM</th>
<th>Baseline</th>
<th>Adrenaline</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (n=29)</td>
<td>0</td>
<td>1/29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:NRVT</td>
<td></td>
</tr>
<tr>
<td>0.5 (n=24)</td>
<td>1/24</td>
<td>3/24</td>
<td>3/24</td>
</tr>
<tr>
<td></td>
<td>1: NSVT</td>
<td>1: NSVT</td>
<td>2: NSVT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2: SVT</td>
<td>1: SVT</td>
</tr>
<tr>
<td>1 (n=32)</td>
<td>0</td>
<td>3/32</td>
<td>2/32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3: NSVT</td>
<td>2: NSVT</td>
</tr>
<tr>
<td>10 (n=31)</td>
<td>0</td>
<td>1/31</td>
<td>3/31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1: SVT</td>
<td>2: NSVT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1: NRVT</td>
</tr>
<tr>
<td>100 (n=19)</td>
<td>0</td>
<td>0</td>
<td>1/19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1: NSVT</td>
</tr>
<tr>
<td>1000 (n=24)</td>
<td>0</td>
<td>0</td>
<td>1/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1: NSVT</td>
</tr>
<tr>
<td>10000 (n=29)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n represents the number of cells used in each experiment. SVT: Sustained VT, NSVT: Non-sustained VT, NRVT: Non-recovered VT.

#### 5.2.5 Bisoprolol testing in HCM-specific hiPSC-CMs

In study II, bisoprolol was used to examine antiarrhythmic effects in HCM-specific hiPSC-CMs. (Figure 15) For this aim, an extracellular solution containing adrenaline was first administered for a minimum of 2 minutes. Then, an extracellular solution containing both adrenaline and bisoprolol was used, and finally an extracellular solution was used with bisoprolol alone. Only 0.5 nM adrenaline was used for the efficacy testing of bisoprolol because all types of arrhythmias were observed at this adrenaline concentration. Initially, 1 µM bisoprolol was tested for antiarrhythmic effects against the 0.5 nM adrenaline in HCMT-CMs. However, this concentration could not significantly reduce the DAD rate or phase 3 EAD rate in the presence of or after wash-out of 0.5 nM adrenaline. (Figure 15 A-B,F-G) The frequency of bursts was reduced by 1 µM bisoprolol during the administration of 0.5 nM adrenaline; however, this effect did not persist after the wash-out of 0.5 nM adrenaline. (Figure 15 C and H) The frequency of QES-EAD events was not reduced by 1µM bisoprolol in the presence or after the wash-out of 0.5 nM adrenaline. (Figure 15 D and I) Although, 1 µM bisoprolol was able to reduce the frequency of VT slightly, it could not completely prevent the occurrence of VT in HCMT-CMs. (Figure 15 E and J) We postulated that 1 µM bisoprolol was an insufficient
concentration to reduce arrhythmias, and thus, we increased the concentration of bisoprolol to 10 µM. The 10 µM bisoprolol reduced the DAD rate in both the presence and after the wash-out of 0.5 nM adrenaline. (Figure 15 A and F) However, these reductions were not observed for the phase 3 EAD rate. (Figure B and G) The frequency of bursts was reduced by 10 µM bisoprolol in the presence of 0.5 nM adrenaline, but was not reduced in wash-out conditions. (Figure 15D and I) The frequency of QES-EAD events and VT were not abolished by 10 µM bisoprolol in the presence or after wash-out of 0.5 nM adrenaline. (Figure 15 D-E and I-J) Similarly, we also examined the efficacy of 1 µM and 10 µM bisoprolol in the presence and after the wash-out of 10 nM adrenaline in HCMM-CMs. The DAD rates were reduced by 10 µM bisoprolol both in the presence and after wash-out of 10 nM adrenaline. (Figure 15 K and O) By contrast, phase 3 EAD rates were increased with 10 µM concentration of bisoprolol in HCMM-CMs. (Figure 15 L and P) The bursts were not observed either in the combination of 10 nM adrenaline and 1 µM bisoprolol or after the wash-out of 10 nM adrenaline, however, bursts were recorded during the combination of 10 nM adrenaline and 10 µM bisoprolol. (Figure M and Q) In addition, the frequencies of QES-EAD events were increased in the presence of both 1 µM and 10 µM bisoprolol during and wash-out condition of 10 nM adrenaline in HCMM-CMs. (Figure 15N and R)
Figure 15. Bisoprolol testing in HCM-specific hiPSC-CMs. (A-E) Frequency quantification of arrhythmias in HCMT-CMs in the presence of 0.5 nM adrenaline (0.5 nM A) and, with the addition of 1 μM bisoprolol (0.5 nM A+1 μM B) or 10 μM bisoprolol (0.5 nM A+10 μM B). (F-J) Frequency quantification of arrhythmias in HCMT-CMs after wash-out of 0.5 nM adrenaline with 0 μM (no B), 1 μM (1 μM B) or 10 μM (10 μM B) bisoprolol. (K-N) Frequency quantification of arrhythmias in HCMM-CMs in the presence of 10 nM adrenaline (10 nM A), and with addition of 1 μM (10 nM A+1 μM B) or 10 μM (10 nM A+10 μM B) bisoprolol. (O-R) Frequency quantification of arrhythmias in HCMM-CMs after the wash-out of 10 nM A with 0 μM (no B), 1 μM (μMB) or 10 μM (10 μM B) bisoprolol.

5.3 Combined action potential and calcium transient recording

5.3.1 Interrelation between action potential and calcium transient

APs were recorded from spontaneously beating hiPSC-CMs simultaneously with a CaT measurement of the same cells used in study III. APs were recorded continuously, whereas
CaTs were only recorded for 30 s. The beginning and end of CaTs recording were marked by synchronization pulses sent from the Ca$^{2+}$ imaging system to the patch clamp system. In other words, once the CaT recording started, synchronization pulses were sent continuously and CaTs were recorded along with AP. Synchronization pulses were halted when the CaTs were stopped recording. The upstroke of AP was followed by an increased $[\text{Ca}^{2+}]_i$, and then $[\text{Ca}^{2+}]_i$ decreased to a minimum diastolic level after $V_m$ reached MDP. Our results revealed that APD90 and CaT90 were $326.3 \pm 4.5$ ms (N=37, n=583) and $790.7 \pm 13.7$ ms (N=37, n=583) respectively in ventricular-like hiPSC-CMs, which implies that CaT90s were ~ 2.4 times longer than APD90. In addition, there was positive correlation between APD90 and CaT90. During the DADs, not all $V_m$ oscillation were characterized by an elevation in $[\text{Ca}^{2+}]_i$. In some cases, no change in intracellular calcium level was observed even though $V_m$ was oscillating. (Figure 16 A-B and D-E) Furthermore, the elevated $[\text{Ca}^{2+}]_i$ was observed in phase 3 EAD. (Figure 16 C and F)

5.3.2 Effects of drugs on action potential and calcium transients

To determine whether $I_{\text{Ca,L}}$ is essential for the spontaneous beating of hiPSC-CMs, 5 μM nimodipine was applied to hiPSC-CMs in extracellular solution. This exposure of nimodipine caused the cessation of both AP and CaT in hiPSC-CMs, which means that spontaneous beating was interrupted. (data not shown) To investigate the effect of $I_{\text{Kr}}$ blockage on both $V_m$ and CaT, 650 nM E-4031 was used. Four major types of responses were observed after exposure to E-4031: (1) the prolongation of APD90 and CaT90 without the occurrence of EAD, (2) the prolongation of both APD90 and CaT90 with phase 2 EADs and (3) the oscillation of $V_m$ and CaT with and without the presence of phase 2/3 EADs, and (4) the cessation of spontaneous beating. (Figure 16 G-L) Notably, the MDP was decreased by E-4031. Furthermore, CaT followed the $V_m$ in phase 2 EAD, and the time gap between $V_m$ and CaT was getting closer during the terminal part of AP repolarization.
Figure 16. Simultaneous recording of the membrane potential ($V_m$) and Ca$^{2+}$ transients (CaTs) from the same hiPSC-CMs. (A) Action potentials (APs) exhibiting delayed after depolarization (DAD) events with a corresponding elevation in CaT (D). (B) APs exhibiting DAD events without a corresponding elevation in CaT (E). (C) APs exhibiting phase 3 early after depolarization (EAD) and corresponding CaT (F). (G) Representative traces of prolongation of AP durations and CaT (J). (H) AP exhibiting phase 2 EAD and CaTs following the $V_m$ trend(K). (I) APs exhibiting $V_m$ oscillation and phase 3 EAD with CaT (L) following the $V_m$ trend. Dashed lines represent 0 mV. Arrows indicate the DAD events, phase 2 EADs, and phase 3 EADs.
6. Discussion

The first two studies focused on the establishment and implementation of hiPSC-CMs obtained from HCM patients carrying MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. These hiPSC-CMs exhibited the disease phenotype, and characteristics differences not only from control hiPSC-CMs, but also between the two HCM-causing mutations. The third study focused on the simultaneous recording of APs and CaTs from the same hiPSC-CMs, and examined the inter-relation between these two parameters.

6.1 Diversity in HCM

There exists great diversity in the clinical phenotypes associated with HCM such as the degree and pattern of hypertrophy (asymmetric, concentric, apical), penetrance, age of onset and clinical course. (Arad et al. 2002) HCM is the most common genetic cardiac disease. Several types of mutations have been identified in association with HCM, including deletions, insertions, missense mutations, and splice site mutations. (Seidman & Seidman 2001; Seidman 2002) The different mutations may yield different biophysical consequences. The initial defect induced by the mutant sarcomeric proteins are diverse, which could be mechanical (actomyosin interaction and cardiac myocyte contractile performance), biochemical (Ca$^{2+}$ sensitivity), bioenergetics (ATPase activity), and/or structural (sarcomere assembly, subcellular localization, and stoichiometry). Although the initial defects are diverse, they converge into a common phenotype of compensatory hypertrophy, fibrosis, and disarray. (Marian et al. 2001) However, the actual mechanism of hypertrophy has not yet been identified. In addition, several studies have suggested the important role of other genetic and/or environment modifiers affecting the hypertrophy. (Marian et al. 1993; Lechin et al. 1995; Perkins et al. 2005) In most cases, HCM is caused by single heterozygote mutations in genes encoding sarcomeric proteins. It has been reported that HCM patients can carry more than one disease causing mutation in the same or different sarcomere genes, resulting to a double or compound heterozygote genotype, e.g., β-MHC and MYBPC (Ackerman et al. 2002). Few cases of homozygous mutations in MYH7, TNNT2 or MYBPC3 associated to HCM have been reported. (Ho et al. 2000; Richard et al. 2000; Zahka et al. 2008) Additionally, HCM caused by triple sarcomere gene mutations have also been reported.(Girolami et al. 2010)

Compared with individuals carrying single-gene mutations, HCM patients with homozygous and heterozygous double or compound mutations exhibit more severe left ventricular hypertrophy, are younger at diagnosis and have higher incidence of SCD. (Kelly & Semsarian 2009) Moreover, HCM patients carrying a sarcomere gene mutation and a mitochondrial mutation have also been reported, and they present more severe cardiac phenotypes (Arbustini et al. 1998). Many genes coding for cardiac sarcomeric proteins are also expressed in skeletal muscles, including β-MYH7, regulatory myosin light chain-2 (MYL2), desmin (DES), and TNNC1. Thus, some of these mutations affect both heart muscle and skeletal muscle. For example, mutations in the MYH7 gene can cause abnormalities both in cardiac and slow-twitch skeletal muscle function yielding cardiomyopathy and/or myopathy. (Homayoun et al. 2011)
Furthermore, gender may be an important factor modifying the phenotypic expression in HCM. Women with HCM are reported to have smaller ventricles, and later onset of disease (Ackerman et al. 2002; Dimitrow et al. 2001). Earlier study of HCM with MYBPC mutations demonstrated that females had more delayed onset of left ventricular hypertrophy compared with males (92% in males and 67% in females in ≤40 years old), female patients were more symptomatic at diagnosis than males, and female patients (who were phenotype-positive) had significantly more frequent heart failure events during the follow-up period. (Terauchi et al. 2014)

Mutations in β-MHC were the first identified cause of familial HCM (Jarcho et al. 1989). Certain mutations in β-MHC are associated with a malignant phenotype characterized by early onset, and a high incidence of premature sudden death (Ackerman et al. 2002; Anan et al. 1994). These features lead to the conclusion that all β-MHC mutations are associated with early onset, and increased risk for SCD. However, clear clinical differences exist, for example, between β-MHC mutations Arg403Gln or Arg719Trp; both are associated with sudden death and heart failure, while mutations Phe513Cys, Leu908Val, or Gly256Glu are associated with severe clinical disease (Marian & Roberts 1995). HCM patients with cTnT mutations display moderate or no significant cardiac hypertrophy despite their malignant prognosis, i.e., high incidence of sudden cardiac death. In cTnT gene missense mutations I79N and R92Q, a deletion mutation ΔE160, and a splice donor site mutation intron 166G→A develop a similar malignant clinical phenotype. (Watkins et al. 1995) Mutations in cTnI (TNNI3) causing HCM have been associated with apical HCM and elderly onset of disease (Kimura et al. 1997). The missense mutations R145G and R145Q reduce the intrinsic inhibitory activity of cTnI without changing its affinity for actin. Similarly, the missense mutation R162W and the deletion mutation ΔK183 reduced the affinity of cTnI for actin without changing its intrinsic inhibitory activity. (Takahashi-Yanaga et al. 2001) A missense mutation L29Q in the cTnC gene (TNNC1) was reported in an HCM patient with late disease onset. HCM with cTnC mutations (A8V, C84Y, and D145E, but not E134D), increased the Ca^{2+} sensitivity of force development in cardiac preparations (Veltri et al. 2017).

Mutation in α-TPM is not common in familial HCM except in Finland. The TPM1-Asp175Asn mutation is one founder mutation causing HCM in Finland, and it accounts for a substantial part of all HCM cases in the Finnish population (Jääskelainen et al. 2013). HCM caused by the TPM1 mutation D175N present a variable phenotype with a good prognosis (Coviello et al. 1997). Although HCM carrying the TPM1-Asp175Asn mutation has been considered benign or represents intermediary risk (Coviello et al. 1997), Hedman and co-workers suggested that this mutation is not entirely benign, and that mutation carriers are at increased risk of fatal arrhythmias at early age (Hedman et al. 2004). Similarly, HCM patients with TPM1-Asp175Asn had a higher rate of SCD in Finland (Table 3) (Jääskelainen et al. 2013). In addition, the thickness of LV in these HCM patients ranges from 15mm to 24 mm (Table 3) (Jääskelainen et al. 2013). The Asp175Asn mutation in TPM1 (used in studies I and II) has also been reported in earlier studies from USA, Japan, and Europe (Jääskelainen et al. 2002). HCM mutations in α-TPM (D175N and E180G) have been proposed to alter thin filament activation (Bing et al. 1997). The α-TPM is a component of sarcomere thin filaments that is also abundantly expressed in cardiac and skeletal muscle. Similarly, skeletal muscles obtained from two HCM
patients carrying the Asp175Asn mutation showed increased Ca\textsuperscript{2+} sensitivity in mutant muscle fibres. (Bottinelli et al. 1998)

Mutations in MYBPC3 are among the most frequent genetic causes of HCM, and these mutations are associated with late onset, moderate hypertrophy, low penetrance, and good prognosis. MYBPC3-Gln1061X mutation is another founder mutation that accounts for a substantial part of all HCM cases in the Finnish population (Jääskeläinen et al. 2013). Furthermore, the MYBPC3-Gln1061X mutation exhibits age-related penetrance with delayed disease onset (Jääskeläinen et al. 2002). Half of the mutations in MYBPC3 are missense, and the remaining half include insertions, deletions, and splice donor/acceptor site mutations that are predicted to cause C-terminal truncation. HCM-causing MYBPC3 mutations are predicted to cause aberrant splicing leading to a frameshift and premature chain termination. However, the truncated peptides have never been identified in human heart tissue. (Marston et al. 2009; Van Dijk et al. 2009) The Gln1061X mutation in MYBPC3 (used in studies I and II) was first reported in Finland (Jääskeläinen et al. 2002) and later found in 2 HCM patients in Poland and in one HCM patient in Netherland. Thus, MYBPC3-Gln1061X mutation causing HCM is uncommon outside Finland.

There is an ongoing debate over the prognostic significance of HCM causing mutations. There are exceptions for every genotype-phenotype association; this impedes for the use of genotyping alone as clinical and prognostic tool for HCM patients. (Ackerman et al. 2002) Some other confounding factors include the variability and phenotypic expression within individual families, small size of many family studies, modifier genes, role of polymorphisms, and other nongenetic factors. Although mutations in MYH7 (R403Q, R453C, G716R, and R719) were initially considered as malignant phenotype, several exceptions have been found such as only modest hypertrophy, mild symptoms of HCM, and no history of SCD. (Ackerman et al. 2002) Thus, these results weaken the assumption that risk of SCD can be associated with certain type of mutation. There is continuous debate over the prognostic significance of mutations causing HCM. There are exceptions in genotype-phenotype association that hinder the use of genotyping alone as a clinical and prognostic tool for individual patients. (Ackerman et al. 2002)

6.2 Disease phenotype in HCM-specific hiPSC-CMs

In studies I and II, we compared the disease phenotypes not only between HCM-specific hiPSC-CMs and WT-CMs, but also between HCM-CMs carrying different mutations. The main aim of study I was to develop in vitro models of HCM using hiPSC-derived cardiomyocytes carrying either the MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. The characterization of these lines included the verification of pluripotency mediated by the reactivation of endogenous pluripotency markers, and the formation of all three germ layers in EBs. Importantly, the presence of TPM1-Asp175Asn or MYBPC3-Gln1061X mutations in the patient-specific hiPSC lines was confirmed. All the hiPSC lines were able to differentiate into functional cardiomyocytes expressing key ion channels and E-C coupling proteins. In addition,
we showed that HCM-specific hiPSC-CMs demonstrate disease phenotypes such as cellular enlargement and abnormal Ca\(^{2+}\) handling which is consistent with the previous modelling of HCM-specific hiPSC-CMs with different mutations in MYH7 (Arg663His and Arg442Gly) (Lan, Andrew S. Lee, et al. 2013; Han et al. 2014). Similar to previous observations (Lan, Andrew S. Lee, et al. 2013; Han et al. 2014; Coppini et al. 2013), the APD90s of cardiomyocytes carrying TMP1-Asp175Asn or MYBPC3-Gln1061X mutation were significantly longer than those of control cardiomyocytes. Additionally, the APD90s of cardiomyocytes carrying TMP1-Asp175Asn mutation were significantly longer than those of cardiomyocytes carrying MYBPC3-Gln1061X mutation as shown in study I. An earlier study demonstrated that isolated cardiomyocytes from HCM patients had markedly prolonged APD, and these patients showed prolonged QTc (corrected QT interval) on ECG (Coppini et al. 2013). Furthermore, there is positive correlation between the degree of left ventricular hypertrophy (expressed by either maximal wall thickness or total number of hypertrophied segments) and QTc. (Dritsas et al. 1992)

In study II, voltage clamp results demonstrated that I\(_{Ca}\) current densities were significantly higher in hiPSC-CMs carrying TMP1-Asp175Asn or MYBPC3-Gln1061X mutation than in control hiPSC-CMs, but I\(_{Ca}\) current densities were not significantly different between these two diseased cardiomyocytes. There are various ionic currents involved in determining APD, and I\(_{Ca}\) is one of the main components (Han et al. 2014). Other potential ionic currents governing the APD include increased late Na\(^{+}\) current (Coppini et al. 2013), decreased I\(_{Kr}\) (Lahti et al. 2012), and reduced I\(_{Ks}\) (Ma et al. 2015). Furthermore, the increased I\(_{Ca}\) densities observed in study II are consistent with previous HCM models (Han et al. 2014; Coppini et al. 2013). By contrast, I\(_{to}\) current densities were significantly lower in hiPSC-CMs carrying TMP1-Asp175Asn or MYBPC3-Gln1061X mutation compared with control hiPSC-CMs, but again no differences were found between mutated hiPSC-CMs (study II). The decreased I\(_{to}\) current densities were consistent with HCM model using isolated human cardiomyocytes (Coppini et al. 2013), but contradicted results with hiPSC-CMs from HCM patients carrying a single missense mutation (R442G) in the MYH7 gene (Han et al. 2014). The remodelling of ion channels plays a crucial role in facilitating the arrhythmias in HCM (Tomaselli & Marbán 1999). Studies I and II showed that HCM-specific hiPSC-CMs exhibited more arrhythmias, including DAD and EAD events compared with control hiPSC-CMs. These DAD and EAD events occurred either alone or in the same cells because the common mechanism for both involves the SR Ca\(^{2+}\) overloading (Volders et al. 1997). Delayed I\(_{Ca,L}\) inactivation and increased late I\(_{Na}\) enhanced CaMKII activity, thus facilitating APD prolongation and arrhythmias. The sustained activation of the CaMKII-dependent signalling pathway plays a critical role in cardiomyocyte remodelling in HCM. (Coppini et al. 2013)

Our results in study I demonstrated the upregulation of α-TPM in hiPSC-CMs carrying TMP1-Asp175Asn or MYBPC3-Gln1061X mutation compared to control hiPSC-CMs. However, an earlier study of hiPSC-CMs carrying mutation in MYBPC3 expressed significantly lower levels of MYBPC protein compared to control. (Tanaka et al. 2014). In study I, the truncated form of MYBPC could not be detected in cardiomyocytes carrying MYBPC3-Gln1061X mutation, but the MYBPC proteins level was reduced, thus confirming the earlier finding of decreased
mutant protein expression. An earlier study of cardiac biopsies from HCM patients carrying mutations in MYBPC3 (W792fs and P955fs), the significant loss of MYBPC3 protein expression was detected, but were unable to detect the truncated proteins. This indicates that approximately 33% reduction in MYBPC3 triggers the hypertrophy and causes contractile dysfunction (Van Dijk et al. 2009). Furthermore, the contractile deficits including higher Ca²⁺ sensitivity of force development and decreased maximal force production in mutant MYBPC3 patients may be either direct or indirect consequence of MYBC haploinsufficiency (Van Dijk et al. 2009). The MYBPC deficiency is associated with higher Ca²⁺-sensitivity, and higher Ca²⁺-sensitivity is consistent with the abnormality in HCM. (Marston et al. 2012) In another study, faster cross-bridge kinetics in HCM patients (MYBPC3 T2604A+C deletion at 2605) results from MYBPC haploinsufficiency. (Hoskins et al. 2010)

HCM patients exhibit a higher incidence of arrhythmias, and this increased arrhythmogenicity was also observed in hiPSC-CMs. In studies I and II, hiPSC-CMs carrying TMP1-Asp175Asn or MYBPC3-Gln1061X mutation had an increased incidence of DAD and EAD events. Clinically, HCM patients with MYBPC3-Gln1061X had higher frequencies of SVT and NSVT, but HCM patients with TPM1-Asp175Asn had a higher frequency of VF (Jääskelainen et al. 2013). However, in study II, the VT-type of arrhythmias were only observed in hiPSC-CMs carrying TMP1-Asp175Asn mutation, not in hiPSC-CMs carrying MYBPC3-Gln1061X mutation. Thus, our finding is in line with the finding from earlier clinical studies that patients with TPM1-Asp175Asn mutation are at increased risk of lethal arrhythmias. The repolarization variability exhibit the repolarization reserve suggesting that the higher the variability, the larger the susceptibility to repolarization-dependent ventricular arrhythmias (Varró & Baczkó 2011). In an early clinical study, the STV of the QT interval was significantly higher in HCM patients compared with healthy individuals (Orosz et al. 2015). In another study, cardiomyocytes isolated from dogs exhibiting VF had increased variabilities in APD50 and APD90 compared to CMs not displaying VF (Sridhar et al. 2008). In study II, VT-type of arrhythmias were only observed in cardiomyocytes carrying TMP1-Asp175Asn mutation. These cardiomyocytes displayed significantly higher repolarization variabilities compared with control cardiomyocytes, which suggests a link between repolarization variabilities and VT-type arrhythmias, and this finding is also in line with earlier observations.

6.3 Effects of adrenergic stimulation in HCM

Adrenaline level in blood circulation increases during exercise and starts to return normal once exercise is stopped. The β-ARs are activated quickly during acute physical activity or emotional activity (Moss et al. 1991), whereas a sustained β-adrenergic response occurs during exercise with a more gradual change in β-adrenergic activation state. Earlier studies reported that the plasma adrenaline concentration is similar between control and HCM groups during resting and exercise (Omodani et al. 1998). The unexpected SCDs occur during or immediately after moderate to severe physical activity. Thus, exercise is considered as an important risk factor of SCD in HCM; however, a decisive role of exercise is not fully understood yet. (van Rijsinge et al. 2011) Clinically, the study of exercise-induced arrhythmia
in HCM is challenging because HCM patients are advised against participating in competitive exercise due to impaired hemodynamics, tolerance during exercise, and an increased risk of lethal arrhythmias. (Elliott et al. 2014)

Study II analysed the role of adrenaline in HCM-specific hiPSC-CMs both during its presence and immediately after washout of adrenaline. Earlier studies with in vitro HCM models focused on using isoproterenol for β-adrenergic stimulation, and demonstrated an increased frequency of arrhythmias in the cells (Knollmann et al. 2003; Lan, Andrew S. Lee, et al. 2013; Han et al. 2014). However, in study II, we used adrenaline for β-ARs stimulation because adrenaline is an endogenous catecholamine whereas isoproterenol is an exogenous agent. Isoproterenol only activates the β-ARs (both β1 and β2) whereas adrenaline can stimulate both α- and β-adrenergic receptors with the same affinity (Brodde & Michel 1999). Adrenaline induces a positive chronotropic and inotropic effect and decrease in APD, mainly via the β-adrenergic pathway (Jakob et al. 1988). β-ARs stimulation differentially modulates different cardiac ion channels and thus fine-tunes AP shape and beating rate. In addition, β-ARs activation influences several ion channels and Ca^{2+} handling on different time scales. Adrenaline shortens the AP by steepening the slope of restitution curve, and the process involves following mechanisms: (1) the inactivation of If, (2) the reduction of inward NCX current and (3) the activation of IKr and IKs. (Taggart et al. 2003) The β-adrenergic signalling leads to a further increase in intracellular Ca^{2+} entry by increasing the mean channel opening time and/or the probability of Ca^{2+} channels opening, which facilitates the Ca^{2+} induce inactivation of Ica,t. (Kamp & Hell 2000). Inward NCX current is reduced due to reduction of the intracellular Ca^{2+} transient because β-ARs agonists induce the phosphorylation of PLB by cAMP-dependent PKA leading to enhanced SR Ca^{2+} reuptake via the SERCA2 pathway (Mattiazzi & Kranias 2014; Mattiazzi & Kranias 2011; Barry & Bridge 1993). In addition to the activation of SERCA2, shortening of APD and corresponding enhanced extrusion of Ca^{2+} by NCX protects the progressive Ca^{2+} overload, which does not usually occur with adrenergic stimulation. The β-ARs stimulation facilitates IKs but its effect on If is controversial; reports have shown increased, decreased, or no effect upon the activation of receptors (Banyasz et al. 2014). The heart/beat rate is increased by β-ARs activation, and β-ARs related increase in IKs shortens the APD in support of faster heartbeats. Furthermore, an adrenergic stimulation-induced increase in beat rate is partly due to the shortening of diastolic duration associated with a faster slope of diastolic depolarization. The β-ARs stimulation increases the intracellular cAMP, which is a second messenger in If modulation that shifts the If-activation curve to more positive voltages, and results in greater activation of If at diastolic potentials (Difrancesco 2010). In addition, the upregulation of Ica,t by β-ARs stimulation via a PKA-dependent mechanism also contributes to the increase in heart rate (Li et al. 2017). β-ARs stimulation also causes the cAMP–dependent phosphorylation of cTnI, which is associated with a decrease in myofilaen Ca^{2+} sensitivity thereby contributing to further enhancement of myocardial relaxation. (Okazaki et al. 1990). Additionally, cardiac hypertrophy and the progression of heart failure involve the reduction or desensitization of β-ARs as well as irregularities in β-adrenergic signalling. Earlier studies demonstrated the reduction in β-ARs density (Choudhury et al. 1996) and a desensitized cardiac β-ARs system (Schumacher et al. 1995) in HCM patients under normal plasma catecholamines levels.
In a previous clinical study using stress/exercise tests, some HCM patients had NSVT and VF episodes, but all HCM patients were asymptomatic (Gimeno et al. 2009). In our study, HCM patients whose hiPSC-CMs showed NSVT were also clinically asymptomatic. However, a transition from NSVT to SVT or NRVT occurred only after the administration of a low concentration of adrenaline in cardiomyocytes carrying the TPM1-Asp175Asn mutation. Xie et al. presented a possible mechanism for this transition via β-ARs stimulation (Xie et al. 2014). SCD can occur in HCM, especially during exercise; however, it does not appear due to result from LV outflow obstruction. (Koga et al. 1984). Although the occurrence of arrhythmias during exercise is not frequent in HCM, the existence of arrhythmias is potential link with the increased risk of SCD (Gimeno et al. 2009).

6.4 Pharmacological treatment in HCM

Medical treatment has primarily been suggested for patients with symptomatic HCM. Pharmacological treatment is considered the initial therapeutic approach in symptomatic HCM patients, drugs are mainly used to treat heart failure and angina symptoms, LV outflow tract obstruction, and arrhythmias. (Gersh et al. 2011) However, there is no specific drug available for HCM, and β-blocker therapy is the initial standard clinical intervention for symptomatic HCM patients because of their negative inotropic effects. (Spoladore et al. 2012) The dose range of β-blockers given to HCM patients is an important factor, and thus the dose needs to be carefully titrated to the maximum tolerated dose on an individual basis for maximum benefit. (Task et al. 2014; Ostman-Smith 2010). Ca^{2+} channel blockers, such as verapamil/diltiazem are prescribed for the treatment of symptoms (angina or dyspnea) in HCM patients who do not respond to β-blockers or who have side effects. If β-blockers or verapamil alone are ineffective, disopyramide, which is titrated up to a maximum tolerated dose may be added. However, disopyramide alone without β-blockers or verapamil is potentially harmful in the treatment of symptoms in HCM patients with atrial fibrillation because it may enhance atrioventricular conduction and increase the ventricular rate during atrial fibrillation. (Gersh et al. 2011; Elliott et al. 2014) For HCM patients for whom medical therapy is ineffective, other treatment options include septal myectomy, dual-chamber pacing or septal ablation. (Task et al. 2014; Spoladore et al. 2012) VF and VT are the primary causes of SCD in HCM, and the prevention of SCD in HCM patients continues to be a major challenge. At present, no antiarrhythmic drugs including β-blockers have been clinically proven to improve survival in HCM patients. (Task et al. 2014) Amiodarone has improved survival in young HCM patients (McKenna et al. 1985), but side effects and the potential toxicity of extended use limit its use in young patients for whom the risk may outweigh the benefit (Cecchi et al. 1998). SCD occurs primarily in asymptomatic or mildly symptomatic patients, and particularly at a young age. The identification of patients at risk of SCD is a fundamental component in disease management. Several potential risk factors for SCD have been reported in patients with HCM including (1) prior personal history of cardiac arrest, VF, or VT; (2) family history of premature SCD, particularly if multiple occurrences; (3) unexplained syncope, NSVT, abnormal blood pressure response to exercise; and (4) extreme left ventricular hypertrophy (> 30 mm). However, all of these risk factors
except cardiac arrest have relatively low positive predictive accuracy (~10%-20%) due to the clinical diversity of the disease. (Gersh et al. 2011) Furthermore, the significance of these risk factors also depends on the age of HCM patients. Some of the risk factors, such as syncope, severe hypertrophy, and history of NSVT are associated with SCD in young patients, whereas the risk of SCD is lower in ≥ 60 year old HCM patients exhibiting risk factors. (Gersh et al. 2011) The presence of single risk factors may be sufficient to warrant ICD placement in many HCM patients, but these decisions need to be individualized with respect to age, the strength of the risk factor, and the risk-benefit of lifelong ICD therapy (Gersh et al. 2011).

In study II, we used bisoprolol to examine its antiarrhythmic efficacy in HCM-specific hiPSC-CMs. First, we used 1 µM bisoprolol in the presence of adrenaline (0.5 nM for HCMT-CMs and 10 nM for HCMM-CMs), but no decrease in the frequency of arrhythmias was observed. An increased dose of 10 µM bisoprolol was then used based on speculation that a higher concentration could limit the frequency of arrhythmias in HCM-specific hiPSC-CMs. However, 10 µM bisoprolol did not produce an antiarrhythmic effect during the administration of adrenaline or after wash-out of adrenaline. Importantly, 1 µM and 10 µM concentration were unable to prevent the initiation or the termination of VT-type arrhythmias. However, earlier studies claimed that β-blockers could reduce arrhythmias (DADs and arrhythmic events) in HCM-specific hiPSC-CMs, which contradict our results (Lan, Andrew S Lee, et al. 2013; Han et al. 2014). The possible reasons for these conflicting results might be differences in mutations causing HCM, and in the study design, including different concentrations and types of β-blockers used and long-term versus the short-term β-blockers administration. Another reason might be that we had categorized the arrhythmias present in hiPSC-CMs, addressed the frequency of each arrhythmias not only during the administration of adrenaline, but also immediately after its wash-out in the presence of β-blockers. This detailed investigation of the effects of β-blockers on different types of arrhythmias was not performed in earlier studies (Lan, Andrew S Lee, et al. 2013; Han et al. 2014). In addition, HCMT-CMs exhibited VT-type arrhythmias, which were also not reported in earlier HCM models using hiPSC-CMs. Our poor antiarrhythmic potency with the β-blocker is similar to findings in clinical studies. One study showed that programmed ventricular stimulation induced SVT or VF in 33% (7/21) of HCM patients carrying the TPM1-Asp175Asn mutation, although 57% (4/7) of these vulnerable HCM patients were undergoing β-blocker therapy (Hedman et al. 2004). Furthermore, β-blocker therapy in HCM demonstrated an improvement in angina, exercise tolerance, and syncope in 60%-80% of patients, but only ~ 40% of patients have sustained symptom improvement. (Task et al. 2014; Spoladore et al. 2012) There is no evidence that drugs administration prevent SCD in HCM.

6.5 Sudden cardiac death and device therapy in HCM

SCD in HCM is most devastating and unpredictable complication; annual mortality rate of HCM is about 1%. Typically, SCD occurs without warnings in asymptomatic or mildly symptomatic young patients (particularly < 25 years of age). Although SCD risk is lower in midlife and beyond; however this does not confer immunity to SCD. (Maron 2010) There are
various clinical factors associated with the risk of SCD, thus all HCM patients should undergo SCD risk stratification, irrespective of whether symptoms are present. The established risk markers are (1) prior personal history of VF or sustained VT; (2) family history of SCD; (3) syncope; (4) NSVT; (5) maximum LV wall thickness; (6) abnormal blood pressure response during exercise. The presence of single risk marker in HCM patients may be sufficient to warrant ICD therapy; however, the decision needs to be individualized with respect to age, strength of risk factor, and risk-benefit of lifelong ICD therapy. (Gersh et al. 2011; Elliott et al. 2014) Pharmacological therapy has not been shown to protect HCM patients from SCD, and only ICD has proven to be effective in terminating life-threatening ventricular tachyarrhythmia in HCM. An early study comparing antiarrhythmic drug therapy and ICD showed that ICD was superior to antiarrhythmic drugs among patients who experienced VF or sustained VT (McAnulty et al. 1997).

There is universal agreement involving the prudence of secondary ICD prevention for HCM patients who survived from VF or episode of sustained VT because of relatively high recurrence of such lethal ventricular arrhythmias in this subgroup. However, selection of ICD candidate for primary prevention is less resolved. (Maron 2010) The decision for ICD placement for primary prevention involves individual clinical judgments, especially when the evidence for risk is ambiguous. (Gersh et al. 2011; Elliott et al. 2014) Nevertheless, ICD-related complications are well documented. Approximately 25% of HCM patients may experience one or more inappropriate shocks as results of sinus tachycardia, atrial fibrillation, or lead malfunction (Maron et al. 2014); the highest rates of up to 40% reported in children and adolescents. (Maron et al. 2013) The ICD in children or young HCM patients inevitably outweigh benefit for SCD prevention, and the psychological burden imposed by ICD and by quality-of-life restriction. Other reported complications of ICDs are infections, haemorrhage/thrombosis, and lead fracture or dislodgment. Thus, it is important to recognize and discuss with patients about potential ICD-related complications. (Elliott et al. 2014; Gersh et al. 2011)

6.6 Relationship between calcium transient and the membrane potential

The $V_m$ and CaT are two important parameters for the proper functioning of cardiomyocytes. Generally, these parameters are investigated independently because incorporating different techniques to acquire both parameters concurrently is challenging. In study III, we recorded $V_m$ and CaT dynamics simultaneously to understand the interrelation between $V_m$ and CaT in hiPSC-CMs. The CaT90 was 2.4 times longer than APD90; there was strong positive correlation between CaT90 and APD90 in hiPSC-CMs. In an earlier study of simultaneous $V_m$ and Ca$^{2+}$ mapping in hiPSC-CMs monolayer, the CaT90s were also reported to be longer than APD90s (approximately 1.2 fold) (Lee et al. 2012), but the difference was less than that observed in our study. This discrepancy might be because the earlier study used low-affinity ratiometric Fura-4F Ca$^{2+}$ dye, whereas we used high-affinity non-ratiometric Fluo-4 Ca$^{2+}$, which artificially prolongs the CaTD (Fast et al. 2004; Kong & Fast 2014). However, the increasing time of CaT is independent of the type of Ca$^{2+}$ dye used in experiments (Fast et al.
2004). DAD and EAD events could occur in the same cells, particularly because spontaneous SR Ca\textsuperscript{2+} release is one of the aspects responsible for occurrence DAD and EAD events (Volders et al. 1997). The increase in [Ca\textsuperscript{2+}], activates the NCX and Ca\textsuperscript{2+}-activated non-selective cation channels, thus DAD events (Ko et al. 2017; Verkerk et al. 2000). This DAD mechanism was clear in our results because an elevation in [Ca\textsuperscript{2+}], occurred that corresponded with an increase in V\textsubscript{m}. However, we observed cases in which [Ca\textsuperscript{2+}], remained unchanged despite that V\textsubscript{m} was abnormally changing. Three possible explanations for this discrepancy are as follow: (1) the [Ca\textsuperscript{2+}] amplitude was reduced by spatial averaging because the amplitude of DAD events without [Ca\textsuperscript{2+}] elevation was significantly lower; (2) Fluo-4 artificially prolonged the CaT90 which overshadowed the change in CaT corresponding to V\textsubscript{m} oscillation observed nearer the terminal repolarization of AP; and (3) the involvement of Ca\textsuperscript{2+}-independent currents promoted the depolarization of V\textsubscript{m}, of which the possible mechanisms is still unknown. Similarly as an earlier study (Mitsunori et al. 2010), the amplitude of DAD was dependent on the amplitude of [Ca\textsuperscript{2+}], elevation in our study. Furthermore, the DAD amplitude also depends on the sensitivity of resting V\textsubscript{m} to changes in [Ca\textsuperscript{2+}], i.e., diastolic Ca\textsuperscript{2+}-voltage coupling gain (Mitsunori et al. 2010). The APDs and CaTs were prolonged concurrently with the pharmacological blockage of I\textsubscript{Kr} by E-4031. In addition, blocking I\textsubscript{Kr} also promoted the EAD events; the CaT followed the V\textsubscript{m} during the EADs. Furthermore, the upstroke of an EAD is generally carried by I\textsubscript{Ca}. The take-off potential of an EAD depends on the complex interplay between the kinetics of I\textsubscript{Ca} and I\textsubscript{Ks} (Chang et al. 2012). APD prolongation in association with the instability and triangulation of APD support the development of phase 2 EAD (Hondegem et al. 2001). Pharmacological intervention or pathophysiological conditions associated with EAD follow one of the following mechanisms (1) a reduction of repolarizing K\textsuperscript{+} currents (I\textsubscript{Kr} and I\textsubscript{Ks}); (2) an increase in the availability of Ca\textsuperscript{2+} current; (3) an increase in NCX current caused by augmentation of [Ca\textsuperscript{2+}], or the upregulation of NCX; or (4) an increase in late Na\textsuperscript{+} current. By contrast, the elevated [Ca\textsuperscript{2+}], during repolarization enhances the NCX current, which could potentially trigger phase 3 EAD events (Mitsunori et al. 2010; Volders et al. 2000). These examples also demonstrate the strong interdependency between V\textsubscript{m} and CaT during phase 2/3 EAD episodes. An earlier study reported that the CaT also faithfully tracked with the V\textsubscript{m} during faster beating frequency and VT (Wu et al. 2005). However, it is possibile that SR Ca\textsuperscript{2+} cycling undergo the intrinsic dynamic dissociation with V\textsubscript{m}. One such example is during VF, during which CaT is no longer associated with V\textsubscript{m}. (Omichi 2004; Wu et al. 2005)
6.7 Limitation of the study

One of the main limitations of studies I-III is the immaturity of the hiPSC-CMs. Generally, these hiPSC-CMs exhibit some fundamental differences compared to adult cardiomyocytes such as the lack of t-tubules, low-level expression of \( I_{K1} \), and weaker contractility (Knollmann 2013). Thus, these hiPSC-CMs do not fully resemble adult cardiomyocytes. Another limitation of this study is that END-2 differentiated cardiomyocytes have different action potential morphologies, and the classification of cardiomyocytes (ventricular-like, atrial-like, and nodal-like) is mainly based on these morphologies, i.e., APD ratios, APA, and \( dV/dT \) (Mummery 2003). The disadvantage of this classification is that cardiomyocytes may not be categorized accurately. In addition, the END-2 differentiation protocol used in studies I-III yields a majority (> 80%) of ventricular-like CMs, whereas only a minority (< 15%) of CMs are atrial-like or nodal-like cardiomyocytes. This lack of specific cell types hinders our understanding of the functioning, drug responses, and arrhythmias in atrial-like and nodal-like cardiomyocytes. Furthermore, hiPSC-CMs exhibit high cell-to-cell and line-to-line variability. The human heart consists of not only myocytes, but also other cell types, such as endothelial cells, smooth muscle cells, and fibroblasts. In addition, there exists crosstalk between the cells. However, hiPSC-CMs established with current differentiation protocols lack other defined cell types and cell-cell communication. Microscopic single hiPSC-CMs cannot completely model the complete macroscopic human heart.

One of the main challenges in disease modelling using hiPSC-CMs is the establishment of appropriate controls. The control hiPSC-CMs used in studies I-II are from completely different genetic/family backgrounds from HCM patients. Thus, examining the results from HCM-specific hiPSC-CMs relative to control hiPSC-CMs might not be the most optimal comparison due to abundant genotypic differences. It is generally argued/suggested that the comparison should be performed between siblings with and without HCM-causing mutation within the same family groups. In other words, the control hiPSC-CMs should have the same genetic background as the mutant hiPSC-CMs. However, in studies in which healthy siblings have been used as controls, only \( \sim 50% \) of the genome is shared between siblings, and thus phenotypic differences could result from DNA variants in the other \( \sim 50% \) of the genome, in addition to the disease-associated mutations. (Musunuru 2013)

Study II raises new questions, and our comprehensive study reveal a previously unknown role of adrenaline in HCM-specific hiPSC-CMs. Unanswered questions include why hiPSC-CMs with different HCM-causing mutations respond differently to endogenous adrenaline and why VT-type arrhythmias are exclusively present in hiPSC-CMs with the \( TPMI-Asp175Asn \) mutation. In this thesis, we were unable to answer these fundamental questions. Moreover, we included two founder mutations associated with HCM patients in studies I-II. These two mutations causing HCM are common in Finland, but infrequent elsewhere. The results obtained from studies I-II may only be specific to these two mutations, and cannot readily be extrapolated to all HCM patients. In addition, technical limitations existed in study III. During APs measurements, the sampling rate was 20 kHz, which is sufficient to measure small changes
in $V_m$. However, the frame-recording interval of 20 ms in Ca$^{2+}$ imaging is not sufficiently fast enough to capture small intracellular dynamics especially, during phase 0 of AP.

### 6.8 Future perspectives

The reprogramming of somatic cells into pluripotent stem cells and subsequent differentiation into specific cell types is a new technique. This emerging technology has some fundamental drawbacks, but the methodology also provides crucial advantages that conventional *in vitro* disease models (e.g., transfected cells and animal cells) can never offer, i.e., it provides patient-specific human cardiomyocytes.

The most problematic aspect of hiPSC-CMs is their immaturity and their resemblance to fetal cardiomyocytes. Thus, it is imperative to enhance the maturity of hiPSC-CMs. One possible method to improve the maturity of hiPSC-CMs is to culture in a 3-dimensional structure. The 3-dimensional culture of hiPSC-CMs results in higher $I_{Na}$ density and upstroke velocity comparable to adult cardiomyocytes (Lemoine et al. 2017). In addition, 3-dimensional culture enhances the metabolic maturation of hiPSC-CMs to a state similar to adult cardiomyocytes (Correia et al. 2017). Furthermore, Shadrin and co-workers introduced the “Cardiopatch” platform for the 3-dimensional culture and maturation of hiPSC-CMs; this platform produces robust electromechanical coupling, consistent H-zones and I-bands, and there is also evidence of t-tubules and M-bands (Shadrin et al. 2017). The existing differentiation methods produce a mixed population of different cell types; the majority of cardiomyocytes are ventricular-like hiPSC-CMs. Several cardiac diseases exist in the atrium (such as atrial fibrillation), SA and AV nodes (such as SA disease, heart block), and the conduction system. Subtype-specific cardiomyocytes enable us to study the functioning, drug response, and mechanism of arrhythmias in those specific cardiomyocytes. Several groups have generated atrial-specific cardiomyocytes (Laksman et al. 2017) and ventricular-specific cardiomyocytes (Lee et al. 2017) to examine atrial-selective pharmacology (Devalla et al. 2015).

The Food and Drug Administration (FDA) withdraws the approved drugs from the market for various reasons, for example cisapride (Glessner & Heller 2002). One of the main reasons for the withdrawal of an approved drug is cardiotoxicity, and drug-induced liver injury is another serious complication of drug therapy. Therefore, it is extremely important to test drugs targeted to different organs prior to their introduction into the market. The “Organs-on-chips”, which include several living organs such as cardiomyocytes, lung, kidney, intestine, hepatocytes, and the blood-brain barrier offers a new advanced drug testing platform, and allow us to analyse different molecular and cellular responses to specific drug. The main goals of the “Organ-on-chips” are to replace animal testing and to create and test the personalized medicine in more effective ways. (Caplin et al. 2015) Our BioMediTech is also trying to develop Body-on-Chip Research (CoEBoC).

One challenging aspect of disease modelling is the establishment of appropriate controls. The most appropriate comparison between control and mutant cell lines can be made when they are both derived from the same parental lines, i.e., the only difference is the presence or absence
of a mutation. With the help of the emerging technology of gene editing, mutant genes can be corrected, and isogenic lines can be established. This advance in genome engineering will not only provide more reliable control lines, but also help us to understand how mutations alter the normal functioning of cardiomyocytes by providing powerful tools for understanding disease mechanisms/pathways.

Undoubtedly, measuring various parameters such as $V_m$, CaT and contraction (mechanical behaviour), simultaneously from the same cardiomyocytes allows a better understanding of the cardiac physiology and pathophysiology. In earlier studies seeking to understand interdependency, only two of these parameters were recorded simultaneously, as in the following example: $V_m$ and CaT (Spencer et al. 2014); CaT and contraction (Ahola et al. 2018); or $V_m$ and contraction. However, these studies always elicit the question of how the other remaining parameter (that was not included) behaves in this particular situation. Thus, recording all three parameters simultaneously would provide a new avenue to investigate arrhythmic behaviour and would provide more predictive results in drug development.
7. Conclusion

The following conclusions can be drawn from the three studies included in this thesis:

- hiPSC-CMs carrying TPM1-Asp175Asn or MYBPC3-Gln1061X mutation exhibit HCM phenotype *in vitro*, including abnormal Ca$^{2+}$ handling, a higher frequency of arrhythmias, and prolonged APD. In regard to their functional and characteristic aspects, HCM-specific hiPSC-CMs were not only different from control hiPSC-CMs, but also between cardiomyocytes carrying different mutations.

- hiPSC-CMs carrying TPM1-Asp175Asn or MYBPC3-Gln1061X mutation respond differently to various concentrations of adrenaline. Bisoprolol, a β-adrenergic antagonist, cannot reduce the arrhythmic events during the administration of adrenaline or after its wash-out.

- The beat rate variabilities and APD variabilities are important factors to earn cardiac disease modelling. The BRVs were increased in HCM-specific hiPSC-CMs compared with control hiPSC-CMs. Additionally, APD variabilities associated with VT-type arrhythmias was observed.

- There is a strong correlation between APD90 and CaT90 in hiPSC-CMs. DADs observed in AP recordings are usually characterized by a similar change in CaT. The CaT is always associated with fluctuation in AP during phase 2/3 EAD events.

- The hiPSC-CM model provides a safe and robust platform to study genetic cardiac diseases, and it is a promising tool for drug screening.
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9. Original Publication
Research Article

Mutation-Specific Phenotypes in hiPSC-Derived Cardiomyocytes Carrying Either Myosin-Binding Protein C Or α-Tropomyosin Mutation for Hypertrophic Cardiomyopathy

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Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disease, which affects the structure of heart muscle tissue. The clinical symptoms include arrhythmias, progressive heart failure, and even sudden cardiac death but the mutation carrier can also be totally asymptomatic. To date, over 1400 mutations have been linked to HCM, mostly in genes encoding for sarcomeric proteins. However, the pathophysiological mechanisms of the disease are still largely unknown. Two founder mutations for HCM in Finland are located in myosin-binding protein C (MYBPC3-Gln1061X) and α-tropomyosin (TPM1-Asp175Asn) genes. We studied the properties of HCM cardiomyocytes (CMs) derived from patient-specific human induced pluripotent stem cells (hiPSCs) carrying either MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. Both types of HCM-CMs displayed pathological phenotype of HCM but, more importantly, we found differences between CMs carrying either MYBPC3-Gln1061X or TPM1-Asp175Asn gene mutation in their cellular size, Ca$^{2+}$ handling, and electrophysiological properties, as well as their gene expression profiles. These findings suggest that even though the clinical phenotypes of the patients carrying either MYBPC3-Gln1061X or TPM1-Asp175Asn gene mutation are similar, the genetic background as well as the functional properties on the cellular level might be different, indicating that the pathophysiological mechanisms behind the two mutations would be divergent as well.

1. Introduction

Hypertrophic cardiomyopathy (HCM) is one of the most common genetic cardiac diseases with worldwide prevalence of 1:500, as well as the most common cause of sudden cardiac death (SCD) among young competing athletes. HCM is inherited in an autosomal dominant pattern. Nevertheless, a large clinical diversity and age-related penetrance are typical for HCM. On the tissue level, HCM is characterized by the disarray of cardiomyocytes (CMs) and fibrosis of cardiac tissue, as well as thickened interventricular septum or free left ventricular wall. Clinical symptoms include arrhythmias, progressive heart failure, and even SCD, but on the other hand the mutation carrier can be completely asymptomatic. Altogether more than 1400 mutations in 11 genes encoding for the sarcomeric proteins have been identified and related to HCM. The majority of the mutations are found either in the β-myosin heavy chain (MYH7) or in the myosin-binding protein C (MYBPC3) genes [1]. In Finland, two founder mutations located in MYBPC3 and α-tropomyosin (TPM1) genes and one common mutation in MYH7 gene together account around 24% of all Finnish HCM cases [2, 3].

Although the genetic information related to HCM has been growing in the recent years due to the development of sequencing technologies, exact information of the disease mechanisms remains unclear. Thus, current medication of the disease is directed toward the symptom relief and there is no specific therapy to prevent the onset or progression of the
disease [1]. Most of the HCM studies have been conducted with model systems, mainly either with transgenic mice or by studying human tissues obtained from surgical myectomy from end-stage HCM patients [4]. However, animal models carry only the mutated gene lacking the rest of the genome and myectomy samples are obtained from patients in the late stage of HCM development. Therefore, the discovery of the human induced pluripotent stem cells (hiPSCs) has offered a new valuable tool to model HCM and other cardiac diseases and to study the underlying disease mechanisms [5]. To date, hiPSCs have already been used to model a variety of cardiac diseases: electrical defects, for example, long-QT syndrome [6–8] and catecholaminergic polymorphic ventricular tachycardia (CPVT) [9, 10] as well as cardiomyopathies including dilated cardiomyopathy (DCM) [11] and HCM [12–14].

Here we have derived hiPSCs from patients carrying two of the Finnish HCM founder mutations either in MYBPC3 (MYBPC3-Gln1061X) or in TPM1 (TPM1-Asp175Asn) gene. We have differentiated the patient-specific hiPSCs into CMs and compared the phenotypes of the diseased and control CMs.

2. Materials and Methods

2.1. Ethical Issues. This study was conducted in accordance with the Ethics Committee of Pirkanmaa Hospital District to establish, culture, and differentiate hiPSC lines (R08070). Skin biopsies for hiPSC establishment were received from the Heart Hospital, Tampere University Hospital, Tampere, Finland. Patients donating skin biopsies signed an informed consent after receiving both oral and written descriptions of the study. The teratoma assay, described in Section 2.3.6, was approved by ELLA-Animal Experiment Board of Regional State Administrative Agency for Southern Finland (ESAVI/6543/04.10.03/2011).

2.2. Generation and Culture of Patient-Specific hiPSC Lines. hiPSC lines were generated from skin's fibroblasts either with Sendai reprogramming vectors OCT4, KLF4, c-MYC, and SOX2 using CytoTune-iPS Reprogramming Kit (Life Technologies Ltd., Paisley, UK) according to the manufacturer’s instructions or by using pMX retroviral vectors OCT4, KLF4, c-MYC, and SOX2 with or without Cre-LoxP site as described earlier [6, 15]. UTA.13602.HCMT, UTA.02912.HCMT, and UTA.04511.WT hiPSC lines were generated by using Sendai vectors and UTA.07801.HCM and UTA.06108.HCM by using pMX retroviral vectors with Cre-LoxP site and UTA.04602.WT was generated by using pMX retroviral vectors without Cre-LoxP site. In the present study, one line of each patient was used. hiPSC lines were derived and cultured on mouse embryonic fibroblast (MEF) feeder cell layers (26000 cells/cm², CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany) in human pluripotent stem cell (hPSC) culture medium consisting of knockout-DMEM (kDMEM, Gibco, Life Technologies Ltd.) supplemented with 20% knockout serum replacement (ko-SR, Gibco, Life Technologies Ltd.), 1% nonessential amino acids (NEAA, Lonza Group Ltd., Basel, Switzerland), 2 mM GlutaMax (Gibco, Life Technologies Ltd.), 50 U/mL penicillin/streptomycin (Lonza Group Ltd.), 0.1 mM 2-mercaptoethanol (Lonza Group Ltd.), and 4 ng/mL basic fibroblast growth factor (bFGF, PeproTech, Rocky Hill, NJ, USA).

2.3. Characterization of hiPSC Lines

2.3.1. Mutation Analysis by Genotyping. DNA samples from the hiPSC lines were prepared with TaqMan Sample-to- SNP Kit (Applied Biosystems, Life Technologies Ltd.) and the presence of MYBPC3-Gln1061X and TPM1-Asp175Asn mutation in the patient-specific hiPSC lines was confirmed by custom TaqMan SNP Genotyping Assays (Applied Biosystems, Life Technologies Ltd.) according to the manufacturer’s instructions. In the genotyping assays, MYBPC3-gene as well as TPM1-gene was amplified with specific primers. Furthermore, the presence of the mutations was assessed with mutation-specific FAM labeled probes. VIC labeled probes were used to assess the presence of the wild type allele. Sequences for the primers and probes used in the assay are listed in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/1684792).

2.3.2. The Expression of Mutant and Wild Type Alleles in hiPSC-Derived CMs. RNA samples were collected and extracted from hiPSC-derived CMs (UTA.04511.WT, UTA.02912.HCMT, UTA.07801.HCM, and UTA.06108.HCM) with Norgen’s Total RNA Purification Plus Kit (Norgen Biotek Corp., Ontario, Canada) according to manufacturer’s instructions. 50–100 ng of RNA was transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Ltd.). The expression of TPM1-Asp175Asn or MYBPC3-Gln1061X mutation on mRNA level in the hiPSC-derived CMs was studied by custom TaqMan SNP Genotyping Assays (Applied Biosystems, Life Technologies Ltd.) similarly as that for genotyping described above. Sequences for the primers and probes used in the assay are listed in Supplementary Table 1.

2.3.3. Immunocytochemistry. Undifferentiated hiPSC colonies were fixed with 4% parformaldehyde (PFA, Sigma-Aldrich, Saint Louis, USA), stained with primary antibodies for Nanog (R&D systems Inc., Minneapolis, MN, USA), OCT4 (R&D systems Inc.), SOX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TRA-1-60 (Millipore, Billerica, MA, USA), and TRA-1-81 (Millipore), and visualized with secondary antibodies as described before [16]. Finally, the cells were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) containing 40,6-diamidino-2-phenylindole (DAPI) for the nuclei staining and imaged with an Olympus IX51 phase contrast microscope equipped with fluorescence optics and Olympus DP30BW camera (Olympus Corporation, Hamburg, Germany).

2.3.4. RT-PCR. The RNA was extracted from the hiPSC lines by NucleoSpin RNA II Kit (Macherey-Nagel GmbH & Co., Düren, Germany) and 500–1000 ng of RNA was transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit
The presence of pluripotency genes Nanog, SOX2, OCT4, and c-MYC and the absence of virally imported exogenes (OCT4, SOX2, c-MYC, and KLF4) were confirmed by RT-PCR. GAPDH was used as an endogenous control. The primer sequences used for detection of Sendai transgenes are described in CytoTune-iPS Reprogramming Kit’s manual (Life Technologies Ltd.).

2.3.5. Karyotype Analysis. The karyotypes of hiPSC lines were studied by G-banding (Medix Laboratories, Espoo, Finland) or by KaryoLite assay [17] (Turku Centre for Biotechnology, University of Turku, Turku, Finland).

2.3.6. Pluripotency Analysis. The pluripotency of hiPSC lines was confirmed in vitro by embryoid body (EB) formation and in vivo by teratoma assay. hiPSCs were removed from feeder cell layer and cultured in suspension to form EBs. The EBs were cultured in EB medium consisting of ko-DMEM supplemented with 20% fetal bovine serum (FBS, Biosera, Boussens, France), 1% NEAA (Lonza Group Ltd.), 2 mM GlutaMax (Invitrogen, Life Technologies Ltd.), and 50 μg/mL penicillin/streptomycin (Lonza Group Ltd.) for 4–6 weeks before RNA extraction. 200 ng of RNA was transcribed to cDNA for the RT-PCR analysis. The presence of all three germ layers, endoderm (AFP, SOX17), ectoderm (SOX1, NESTIN, and Musashi), and mesoderm (KDR, alpha cardiac actin), was studied with RT-PCR.

For in vivo pluripotency assay, hiPSCs were injected under the testis capsule of nude mice and the formed teratomas were collected and fixed with 4% PFA 8 weeks after the injection. Teratomas were embedded in paraffin, cut in sections, and stained with haematoxylin and eosin.

2.4. Differentiation of Cardiomyocytes. hiPSCs were differentiated into CMs by coculturing with mouse visceral endodermal-like cells (END-2) (Hubrecht Institute, Utrecht, Netherlands) as described before [18]. After 15–30 days beating areas were cut from cocultures and dissociated into single cells in EB medium by Collagenase A (Roche Diagnostics, Mannheim, Germany) as described earlier [18] and plated on 0.1% gelatin-coated cover slips or well plates for further analysis.

2.5. Characterization of hiPSC-Derived Cardiomyocytes

2.5.1. Immunocytochemistry and Image Analysis. Dissociated CMs were fixed with 4% PFA and stained with Troponin T (cTnT, 1:2000, ab64623, Abcam, Cambridge, MA, USA), MYBP (1:400, sc-166081, Santa Cruz Biotechnology), and TPM1 (1:200, sc-73225, Santa Cruz Biotechnology) primary antibodies, followed by labeling with secondary antibodies. Images were obtained with Olympus IX51 phase contrast microscope equipped with fluorescence optics and Olympus DP308W camera (Olympus Corporation) or with Zeiss AxioScope A1 fluorescent microscope and Zeiss AxioCam MRC5 camera (Carl Zeiss, Jena, Germany). Size of the Troponin T stained CMs was analyzed from 46 to 50 CMs in each cell line by in-house made software (unpublished method). CMs were analyzed from pictures obtained with Olympus IX51 phase contrast microscope. The proportion of multinucleated CMs was determined from the same images (46–50 CMs/cell line).

2.5.2. Ca2+ Imaging. The clusters of CMs were cut, dissociated, plated on 0.1% gelatin-coated coverslips, and cultured for 1, 3, and 6 weeks. To study the Ca2+ handling properties of hiPSC-derived CMs, cells were loaded with 4 μM Fura-2 AM (Molecular Probes, Life Technologies Ltd.) for 30 minutes in perfusate medium. The perfusate medium consisted of (in mM) 137 NaCl, 5 KCl, 0.44 KH2PO4, 20 HEPES, 4.2 NaHCO3, 5 D-glucose, 2 CaCl2, 1.2 MgCl2, and 1 Na-pyruvate dissolved in H2O. pH of the perfusate medium was adjusted to 7.4 with NaOH. The coverslip, containing the dissociated hiPSC-derived CMs, was mounted to an RC-25 recording chamber and continuously perfused with perfusate medium preheated to 35-36°C by an SH-27B inline-heater controlled by a TC-324B unit (all from Warner Instruments Inc., Hamden, USA). The perfusion was controlled by a gravity driven VC2 application system (ALA Scientific Instruments Inc., NY, USA). Coverslip was perfused for 15 minutes for Fura-2 AM deesterification before experimental recordings. Ca2+ handling of spontaneously beating CMs was imaged with an inverted IX70 microscope using UApolo340 x20 air objective (Olympus Corporation) and ANDOR iXon 885 CCD camera (Andor Technology, Belfast, Northern Ireland) synchronized with a Polychrome V light source by a real time DPS control unit. TILLvision® or Live Acquisition software (TILL Photonics, Munich, Germany) was used for recording. Fura-2 AM was excited at 340 nm and 380 nm light and the emission was recorded for 10–30 seconds at 505 nm.

For Ca2+ imaging analysis, single beating CMs were selected as regions of interests and background noise, recorded from a cell-free area in the same coverslip, was subtracted before further processing. Data is presented as ratios of 340/380 nm (F340/F380). The spontaneously beating CMs were divided into five different rhythm categories based on the abnormalities observed in their Ca2+ signals: normal beating with regular peaks (normal); more than three peaks which do not return to the baseline (oscillation); signals with small or middle sized amplitude events in the beginning, in the end, or in between two Ca2+ spikes (low/middle peaks); two or three peaks which do not return to the baseline (double peaks); Ca2+ spikes with prolonged rise or decay time (plateau abnormality). In the low/middle peaks category, the small amplitude was at least 10% from the preceding Ca2+ spike amplitude. Full-length, 10–30 seconds long recordings were analyzed, while most of the analyzed recordings were 12 seconds long. The distribution of CMs in different categories is presented for each cell lines separately.

2.5.3. Electrophysiological Measurements: Recording and Analysis of Action Potentials. The action potentials (APs) were recorded by conventional patch clamp [19] in perforated patch configuration using Amphotericin B [20] in final concentration of 0.24 mg/mL [8]. Data acquisition was
Table 1: TaqMan assays used in qRT-PCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description/alias</th>
<th>Function</th>
<th>TaqMan assay ID</th>
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<tbody>
<tr>
<td>EEF1A1</td>
<td>Eukaryotic translation elongation factor 1 alpha 1</td>
<td>Housekeeping gene</td>
<td>Hs00265885_gl</td>
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<tr>
<td>GAPDH</td>
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<td>Troponin T</td>
<td>Sarcomeric gene</td>
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<td>Myosin heavy chain 6</td>
<td>Sarcomeric gene</td>
<td>Hs0101425_m1</td>
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<td>α-actinin 2</td>
<td>Sarcomeric gene</td>
<td>Hs0013809_m1</td>
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<td>TPM1</td>
<td>α-tropomyosin</td>
<td>Sarcomeric gene</td>
<td>Hs01065966_ml</td>
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<tr>
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<td>Myosin-binding protein C</td>
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<td>Calsequestrin</td>
<td>Ca2+ binding protein in SR</td>
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<tr>
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<td>Ryanodine receptor 2 (cardiac)</td>
<td>Ryanodine receptor</td>
<td>Hs00892883_ml</td>
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Conducted using Axon Series 200B patch-clamp amplifier connected to Digidata 1440a AD/DA converter driven by pCLAMP 10.2 software (all from Molecular devices LLC). On the day of use, the coverslips containing dissociated hiPSC-derived CMs were transferred to RC-24N recording chamber (Warner Instruments Inc.) and mounted on an inverted Olympus IX71 microscope (Olympus Corporation). The patch electrodes had tip resistance of 3.0–3.5 MΩ and contained the following intracellular solution (in mM): 132 KMeSO4, 20 KCl, 1 MgCl2, and 1 CaCl2 (pH was adjusted to 7.2 with KOH). The extracellular solution contained (in mM) 143 NaCl, 4.8 KCl, 1.8 CaCl2, 1.2 MgCl2, 5 glucose, and 10 HEPES (pH was adjusted to 7.4 with NaOH). The preheated extracellular solution was continuously perfused with similar setup compared to what is presented in Section 2.5.2. Patch pipettes (Harvard Apparatus Ltd., Holliston, MA, USA) were freshly prepared using PC-10 micropipette puller and then flame-polished with MF-830 microforge (both from Narishige Int., Tokyo, Japan).

APs were recorded in the gap-free mode in the current clamp from the spontaneously beating hiPSC-derived CMs. Current-clamp recordings were digitally sampled at 20 kHz and filtered at 2 kHz using low pass Bessel filter on recording amplifier. Beats per minute (BPM), AP duration (APD90 and APD50), AP amplitude (APA), and maximum diastolic potential (MDP) were analyzed from the recorded APs by using Origin 9.1 (OriginLab Corp., Northampton, USA). Only ventricular-like waveforms are presented here to avoid any biasness among different hiPSC lines. The ventricular-like CMs were characterized by APD90/APD50 < 1.3 and APA > 90 mV.

2.5.4. Real-Time qRT-PCR Analysis. After one week of culture, dissociated CMs were collected into a lysis solution buffer of CellsDirect One-Step qRT-PCR Kit (Life Technologies Ltd.) according to the manufacturer’s instructions. Two replicate samples were collected and stored at −70 °C until the DNase I digestion and reverse transcription-specific target amplification (RT-STA) by using CellsDirect One-Step qRT-PCR Kit. Real-Time qPCR was performed with Biomark HD system (Fluidigm Corp., San Francisco, USA) according to the manufacturer’s instructions. The TaqMan assays (Life Technologies Ltd.) used in the qRT-PCR are collected in Table 1. All samples were analyzed in duplicate and the fold changes were calculated by the 2−ΔΔCT method [21]. EEF1A1 and GAPDH genes were used as endogenous control genes and UTA.0451.WT cell line was used as a calibrator.

2.5.5. Western Blot. hiPSC-derived CMs were lysed in M-PER protein extraction reagent (Thermo Scientific, Life Technologies Ltd.), supplemented with complete protease inhibitor cocktail (Roche Diagnostics). The protein concentration was quantified with BCA protein assay kit (Thermo Scientific, Life Technologies Ltd.). 10 µg of protein was run to 4–15% mini-PROTEAN TGX precast polyacrylamide gel
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(Bio-Rad, Hercules, CA, USA) and transferred to PVDF membrane (Amersham Hybond-P, GE Healthcare, Little Chalfont, UK). Membranes were blocked with 5% milk for 1 h at RT and proteins were stained with MYBPC (1:1500, sc-166081, Santa Cruz Biotechnology), cTnT (1:2000, ab64623, Abcam), TPM1 (1:200, sc-73225, Santa Cruz Biotechnology), or β-actin (1:1000, sc-47778, Santa Cruz Biotechnology) primary antibodies overnight at +4 °C. Horseradish peroxidase (HRP-) conjugated polyclonal rabbit anti-mouse (DAKO, P0260) and rabbit anti-goat IgG (Santa Cruz Biotechnology, sc-2922) were used as secondary antibodies. Stained proteins were detected by using Clarity ECL substrate (Bio-Rad) and visualized by Molecular Imager ChemiDoc XRS+ (Bio-Rad). ImageJ software (National Institutes of Health, USA) was used to compare the expression of MYBPC, cTnT, and TPM1 with the β-actin expression from the same cell line.

2.6. Statistical Analysis. For statistical analysis, control cell lines and cell lines in each mutation were combined in groups: UTA.04602.WT and UTA.04511.WT hiPSC lines in WT-CM group, UTA.02912.HCMT and UTA.13602.HCMT in HCMT-CM group, and UTA.07801.HCMM and UTA.06108.HCMM in HCMM-CM group. Mann-Whitney U test with Bonferroni’s correction was used to analyze the differences between WT-, HCMT-, and HCMM-CMs in cell size distribution, proportion of multinucleated CMs, and Ca2+ imaging experiments as well as in gene expression analysis. For the statistical comparison between the three groups, one-way ANOVA followed by Tukey test was used for the patch-clamp result analysis. p < 0.05 was considered statistically significant. All error bars are presented as standard error of the mean (SEM).

3. Results

3.1. hiPSCs Were Derived from HCM Patients with Different Backgrounds. We derived hiPSCs from four patients carrying a HCM causing mutation either in TPM1 (TPM1-Asp175Asn) or in MYBPC3 (MYBPC3-Gln1061X). UTA.13602.HCMT and UTA.02912.HCMTs carry TPM1-Asp175Asn and UTA.07801.HCMM and UTA.06108.HCMM in HCMM-CM group. The hiPSC lines and their mutations and abbreviations, used below, are presented in Table 2. UTA.13602.HCMT (46, XX) is derived from a 48-year-old female, whose mother died suddenly at the age of 51. Our patient has had one collapse at the age of 20 with normal heart structure, but later slight thickening of septum (16 mm) has been observed. Currently she is not on medication due to low blood pressure. The pluripotent characteristics of the hiPSC-lines used were assessed (Figure 1 and Supplementary Figures 1–5). UTA.04602.WT cell line has been characterized earlier [22]. All the lines formed colonies, which expressed proteins and genes typical for hPSCs. The virally transferred exogenous genes were silenced and karyotypes of the hiPSC lines were normal. The pluripotency of hiPSC lines was proven in vitro by EB formation or in vivo by teratoma formation. The presence of TPM1-Asp175Asn and MYBPC3-Gln1061X mutations in the patient-specific hiPSC lines was confirmed by custom TaqMan SNP Genotyping Assays (Supplementary Figure 6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell line</th>
<th>Mutation</th>
<th>Name in Figure 2(f)</th>
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<tbody>
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<td>WT</td>
<td>UTA.04602.WT</td>
<td>—</td>
<td>WT1</td>
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<tr>
<td></td>
<td>UTA.04511.WT</td>
<td>—</td>
<td>WT2</td>
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<td>MYBPC3-Gln1061X</td>
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</table>

Table 2: The hiPSC lines and their mutations and abbreviations used in the study.

The presence of MYBPC3-Gln1061X mutations in the patient-specific hiPSC lines has been implanted. UTA.06108.HCMM (46, XY) is derived from a 55-year-old male whose father died suddenly at the age of 36 and uncle at the age of 38. Our patient has been asymptomatic with maximal septal thickness of 22 mm on echocardiogram. He is not on medication due to low blood pressure. Control hiPSC lines were derived from healthy individuals: UTA.04602.WT (46, XX) from a 56-year-old female and UTA.04511.WT (46, XY) from a 34-year-old male.

The pluripotent characteristics of the hiPSC-lines used were assessed (Figure 1 and Supplementary Figures 1–5). UTA.04602.WT cell line has been characterized earlier [22]. All the lines formed colonies, which expressed proteins and genes typical for hPSCs. The virally transferred exogenous genes were silenced and karyotypes of the hiPSC lines were normal. The pluripotency of hiPSC lines was proven in vitro by EB formation or in vivo by teratoma formation. The presence of TPM1-Asp175Asn and MYBPC3-Gln1061X mutations in the patient-specific hiPSC lines was confirmed by custom TaqMan SNP Genotyping Assays (Supplementary Figure 6).

3.2. Mutation-Specific HCM Phenotypes Were Observed in hiPSC-Derived Cardiomyocytes. All the cell lines used in the present study differentiated into cardiomyocytes similarly. Beating aggregates were formed 14–20 days after the initiation of coculturing with END-2 cells and there was no difference between the lines when the beating areas appeared. After cardiac differentiation, beating clusters were dissociated into single cells and cultured for 1, 3, and 6 weeks. The differences in the cell sizes and Ca2+ handling properties between two different mutations and control cells were analyzed in each time point. When comparing different types of CMs, HCMM-CMs were significantly larger than HCMT-CMs and WT-CMs in all three time points (Figures 2(a) and 2(b), n = 96–100, p < 0.005 in all cases). The enlargement of HCMT-CMs was detected after three weeks of culture when they were significantly larger than WT-CMs (p < 0.005). Generally, the size of the hiPSC-derived CMs in all groups increased when the cells cultured for three weeks. Within each group, there were no differences in cell sizes between three and six weeks, except with HCMT-CMs, which seemed to be smaller in size 6-week time point. However, during 6 weeks of culture, cell types other than CMs had the tendency to overgrow the CM culture, which might have affected the cellular enlargement. This phenomenon was
Figure 1: Characterization of UTA.13602.HCMT cell line. (a) The hiPSCs formed colonies expressing Nanog, OCT4, SOX2, TRA-1-60, and TRA-1-81. Scale bars: 200 μm. (b) The virally transferred Sendai exogenes, exo-OCT4 (483 bp), exo-KLF4 (410 bp), exo-SOX2 (451 bp), and exo-c-MYC (532 bp), were silenced in the hiPSCs. + indicates positive controls, for which RNA was extracted from cells 1 week after transduction. hiPSCs expressed endogenous Nanog (287 bp), OCT4 (144 bp), REX1 (306 bp), SOX2 (151 bp), and c-MYC (328 bp). GAPDH (302 bp) was used as a housekeeping control. (c) The hiPSC line was karyotypically normal, 46 XX. (d) The pluripotency of hiPSCs was confirmed by in vivo teratoma assay, in which hiPSCs formed all three germ layers (mesoderm, endoderm, and ectoderm).
Figure 2: Continued.
Figure 2: The cell size and Ca\textsuperscript{2+} handling of hiPSC-derived CMs after 1-, 3-, and 6-week culture as single cells. (a) Representative images of WT-CMs (WT), HCMT-CMs (HCMT), and HCMM-CMs (HCMM) stained with antibodies for cTnT (red) and MYBPC (green) proteins. Scale bars are 100 μm. (b) The size of the HCMM-CMs was significantly larger in all three time points when compared to WT- and HCMT-CMs (\(\text{p}<0.005\) when compared to WT-CMs or HCMT-CMs in the 1-week time point, \(\text{p}<0.005\) when compared to WT-CMs or HCMT-CMs in 3-week time point, and \(\text{p}<0.005\) when compared to WT-CMs or HCMT-CMs in 6-week time point). HCMT-CMs were significantly larger than WT-CMs in 3-week time point (\(\text{p}<0.005\) when compared to WT-CMs, \(n=100\), except in HCMT 6w \(n=96\).) (c) The proportion of the multinucleated CMs was significantly higher in HCMT-CMs than in WT-CMs and HCMM-CMs when both cell lines and all time points were combined (in statistical analysis \(n=6\), *\(p<0.05\)). The averages of multinucleated CMs were determined from the same ca, whose sizes and \(n\) numbers are presented in (b). (d) Significantly more CMs with Ca\textsuperscript{2+} handling abnormalities were observed in HCMT-CMs than in WT-CMs and HCMM-CMs when both cell lines and all time points were combined for each group (in statistical analysis \(n=6\), *\(p<0.05\)). The proportions of CMs with abnormalities in their Ca\textsuperscript{2+} handling were determined from the same Ca\textsuperscript{2+} imaging results presented in (f). The total \(n\) numbers of the analyzed CMs are presented in (f). (e) Representative images of Ca\textsuperscript{2+} rhythm categories. (f) Distributions of hiPSC-derived CMs in different Ca\textsuperscript{2+} rhythm categories (e) in each time point. WT1 = UTA.04602.WT, WT2 = UTA.04511.WT, HCMT1 = UTA.02912.HCMT, HCMT2 = UTA.13602.HCMT, HCMM1 = UTA.07801.HCMM, and HCMM2 = UTA.06108.HCMM.

3.3. Action Potential Characteristics of WT and HCM hiPSC-Derived Cardiomyocytes. The spontaneous action potentials were recorded from the beating hiPSC-derived CMs to establish the electrophysiological baselines. Most of the cells (>80%) were ventricular-like CMs in all the hiPSC lines studied. For this reason, only ventricular-like waveforms are presented here. We first analyzed the percentage of the arrhythmias in each cell line (Figures 3(a)–3(f)) and found similar percentage in both cell lines within the groups (UTA.04602.WT (13%) versus UTA.04511.WT (15%), UTA.02912.HCMT (42%) versus UTA.13602.HCMT (47%), and UTA.07801.HCMM (50%) versus UTA.06108.HCMM (50%)). Based on the percentage of the arrhythmias, we combined hiPSC-derived CMs into groups (WT-CM, HCMT-CM, and HCMM-CM) for further analysis.

Both HCMT-CMs and HCMM-CMs had more arrhythmic events including delayed after depolarizations (DADs) and early after depolarizations (EADs) when compared to the WT-CMs (WT-CM (14%), HCMT-CM (45%), and HCMM-CM (50%)). We quantified the occurrence of DADs in hiPSC-derived CMs as a rate (DADs/min) calculated as total number of DADs/total number of APs. We found that the DAD rate in HCMM-CMs was significantly higher than in WT-CMs (Figure 3(g), \(p<0.005\)).
Figure 3: Arrhythmogenic events (DADs and EADs) were observed in HCM-CMs. (a)–(e) Representative recordings of control hiPSC-derived CMs (WT) and hiPSC-derived CMs carrying TPM1-Asp175Asn (HCMT) or MYBPC3-Gln1061X (HCMM) mutations. Typical DADs (arrows) are presented in (b) and (c) and EADs (arrows) in (d) and (e) for HCMT-CMs and HCMM-CMs, respectively. Scale bars represent 40 mV and 5 seconds, respectively. Scale bars in (a) are representative for (b) and (c), and scale bars in (d) are representative for (e). (f) Distribution of CMs exhibiting arrhythogenic events in each cell line. (g) DAD rate was significantly higher in HCMM-CMs than in WT-CMs (∗∗p < 0.005).
Table 3: AP properties of ventricular-like CMs derived from control hiPSC lines (WT) and from hiPSC lines carrying TPM1-Asp175Asn (HCMT) or MYBPC3-Gln1061X (HCMM) mutations. In the results, the data of each group is comprised from two separate cell lines.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Beating rate (BPM)</th>
<th>APD$_{50}$ (ms)</th>
<th>APD$_{90}$ (ms)</th>
<th>APA (mV)</th>
<th>MDP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>43</td>
<td>58.1 ± 2.3</td>
<td>277.3 ± 13.0</td>
<td>323.6 ± 13.9</td>
<td>119.5 ± 1.1</td>
<td>−76.8 ± 0.8</td>
</tr>
<tr>
<td>HCMT</td>
<td>71</td>
<td>48.4 ± 1.5**</td>
<td>372.3 ± 13.2**</td>
<td>433.1 ± 14.0**</td>
<td>121.2 ± 1.1</td>
<td>−75.8 ± 0.7</td>
</tr>
<tr>
<td>HCMM</td>
<td>54</td>
<td>47.1 ± 1.8**</td>
<td>319.5 ± 13.7$^s$</td>
<td>377.6 ± 15.0$^*,s$</td>
<td>124.3 ± 1.4$^*$</td>
<td>−77.9 ± 0.8</td>
</tr>
</tbody>
</table>

$^*$HCMT or HCMM versus WT.
$^s$HCMM versus HCMT.
$^s$ or $^*$p < 0.05 and $^{**}$p < 0.005.

The average APD at 50% repolarization (APD$_{50}$) and 90% repolarization (APD$_{90}$) of HCMT-CMs was significantly longer than those of the WT-CMs (APD$_{50}$ (p < 0.005) and APD$_{90}$ (p < 0.005)) and HCMM-CMs (APD$_{50}$ (p < 0.05) and APD$_{90}$ (p < 0.05)) (Table 3). APD$_{90}$ of HCMM-CMs was significantly longer than that of the WT-CMs (p < 0.05) (Table 3). Furthermore, the beating rates of both HCMT-CMs and HCMM-CMs were significantly lower than in WT-CMs (WT-CM versus HCMT-CM (p < 0.05) and WT-CM versus HCMM-CM (p < 0.05)). In addition, the APA of HCMM-CMs was significantly higher than in the WT-CMs (p < 0.05). However, no significant differences were found for the MDP between any groups (Table 3).

3.4. Differences in the Gene Expression Profiles of hiPSC-Derived Cardiomyocytes. Dissociated hiPSC-derived CMs were cultured for one week before qRT-PCR analysis was performed. The results are presented in Figure 4. The expression of sarcomeric genes MYBPC3, TNNT2, ACTN2, TTN, MYL7, and MYL9 was significantly higher in both HCMT-CMs and HCMM-CMs than in the WT-CMs (p < 0.005 in all cases). The expression of TPM1 and TNNC1 was significantly increased only in the HCMM-CMs when compared to WT-CMs (p < 0.005 in both cases). On the other hand, the expression of MYH6 was on the same level in all hiPSC-derived CMs. Moreover, the expression of some sarcomeric genes (TNNT2, ACTN2, TNNC1, TTN, MYL7, and MYL9) was significantly higher in the HCMM-CMs than in the HCMT-CMs (p < 0.005 in other than TNNT2 and MYL9 p < 0.05). The expression of natriuretic peptide A (NPPA) was similar in all hiPSC-derived CMs while the expression of natriuretic peptide B (NPPB) was increased in HCMT-CMs and HCMM-CMs when compared to WT-CMs (p < 0.005 in both cases). Nodal marker HCN4 was also significantly increased in both HCMT-CMs when compared to WT-CMs (p < 0.05 for HCMT-CMs versus WT-CMs and p < 0.005 for HCMM-CMs versus WT-CMs). Further, the highest expression of potassium channel KCNQ1 and sodium channel SCN5A as well as sodium calcium exchanger SLCA8A1 was observed in HCMM-CMs (p < 0.005 when compared to WT-CMs in all cases).

We found differences also in the expression of genes related to the Ca$^{2+}$ handling. The expression of CACNA1C and PLN was increased in both HCMT-CMs (p < 0.05 for CACNA1C and p < 0.005 for PLN) and HCMM-CMs (p < 0.005 in both cases) when compared to WT-CMs while the expression of ATP2A2 and ITTPR2 was on the same level in all hiPSC-derived CMs. However, the expression of CASQ2 and RYR2 was significantly higher in HCMT-CMs and HCMM-CMs when compared to WT-CMs (p < 0.005 in all cases). Moreover, the expression of RYR2 was almost six times higher in the HCMT-CMs than in the WT-CMs (p < 0.005) and around three times higher than in HCMT-CMs (p < 0.005).

3.5. Truncated MYBPC Protein Was Not Detected in hiPSC-Derived Cardiomyocytes Carrying MYBPC3-Gln1061X Mutation. Both wild type and the mutant TPM1 mRNA were present in HCM-CMs carrying the TPM1-Asp175Asn mutation while hiPSC-derived control CMs expressed only wild type mRNA. However, in hiPSC-CMs carrying the MYBPC3-Gln1061X mutation the mutant mRNA was not clearly detected (Supplementary Figure 7). At a protein level all hiPSC-derived CMs expressed MYBPC, cTnT, and TPM1 (Figures 5(a) and 5(b)). The truncated MYBPC protein (predicted size: 116 kDa) was not detected in HCMM-CMs with western blot analysis (Figure 5(b)). However, the expression level of total MYBP was slightly reduced in HCMM-CMs when compared to WT-CMs (Figure 5(c)). In addition, the expression of cTnT and TPM1 was elevated in both HCM-CMs (Figure 5(c)).

4. Discussion

Here we have analyzed characteristics of hiPSC-derived HCM-CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X gene mutation. The size of the MYBPC3-Gln1061X CMs was significantly larger than that of TPM1-Asp175Asn CMs, while the CMs carrying the latter mutation had significantly more abnormal Ca$^{2+}$ transients. Additionally, CMs with TPM1-Asp175Asn mutation had significantly more prolonged action potentials. However, both types of HCM-CMs had increased amount of arrhythmogenic events (DADs and EADs) in electrophysiological recordings when compared to control CMs. In addition to morphological and functional differences, also gene expression profiles were different between CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X gene mutation.

Genetic HCM is primarily due to mutations in sarcomeric genes, while changes at the cellular level include disturbed Ca$^{2+}$ metabolism and decreased contraction force generation in addition to enlarged cell size [23]. To our knowledge,
<table>
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<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>WT</th>
<th>HCMT</th>
<th>HCMM</th>
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<tbody>
<tr>
<td>TPM1</td>
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<tr>
<td>MYBPC3</td>
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<td>TNNT2</td>
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<td>ACTN2</td>
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<td>MYH6</td>
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<td>NPPA</td>
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<td>NPPB</td>
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<tr>
<td>HCN4</td>
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<tr>
<td>KCNQ1</td>
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<tr>
<td>SCN5A</td>
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<tr>
<td>SLC8A1</td>
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<tr>
<td>CACNA1C</td>
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<tr>
<td>PLN</td>
<td></td>
<td></td>
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<tr>
<td>ATP2A2</td>
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Figure 4: Continued.
total of three reports studying the characteristics as well as the pathophysiological mechanisms of the HCM by using the patient-specific hiPSCs have been published [12–14]. In two of these publications, the mutation is located in the MYH7 (MYH7-R663H or MYH7-R442G) [12, 13], whereas, in the most recent publication, the hiPSCs were derived from three HCM patients, from whom one carried the MYBPC3-999-1004del2, while the other mutations were unknown [14].

In addition to these three publications, hiPSC-derived CMs carrying MYBPC3 mutations have been used in one study where the effects of serum on the phenotype of neonatal rat CMs as well as hiPSC-derived CMs have been explored [24]. hiPSC-derived CMs carrying MYBPC3 mutation were used only when studying the effects of serum on the cellular enlargement [24]. In this current study, we obtained hiPSCs from HCM patients carrying either the MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. We believe that this is the first report where hiPSC-derived CMs carrying different gene mutations have been compared in the same study with similar experimental settings.

Our HCM-CMs demonstrated cellular enlargement similarly to previous HCM studies with hiPSCs [12–14]. However, we observed a significant difference in the cellular enlargement between the two HCM mutations. CMs carrying the MYBPC3-Gln1061X mutation presented more pronounced and earlier cellular enlargement than CMs carrying the TPM1-Asp175Asn mutation. In a previous study, serum has been shown to mask hypertrophic phenotype of the CMs with mutations in the MYBPC3 [24]. The CMs from the HCM patients were larger in serum-free conditions without any external stimuli, while the serum seemed to increase the cellular enlargement in WT-CMs but not in CMs with HCM mutations [24]. We used 20% serum in our CM culture medium that did not seem to mask the cellular enlargement with our CMs. Already after one week of culture, CMs carrying MYBPC3-Gln1061X mutation were significantly larger than the WT-CMs. The enlargement of CMs carrying TPM1-Asp175Asn mutation was detectable only after three weeks of culture. In the previous clinical studies, as well as in the patient data analyzed in this current study, the hypertrophy has been in the same range in patients carrying either of these two mutations [2]. Therefore, these differences in CM size between the two mutations do not correlate with the extent of clinical hypertrophy.

The higher Ca²⁺ sensitivity, observed in animal models and myectomy samples, has been suggested to be a common feature for all HCM mutations [25, 26]. In the previous studies with hiPSC-derived CMs, irregularities in Ca²⁺ transients have been observed in the MYH7-R663H and the MYH7-R442G mutations [12, 13]. The higher Ca²⁺ sensitivity has been related to lower phosphorylation levels of the MYBPC and the Troponin I proteins and the difference could at least partly be explained by hypophosphorylation of the sarcomeric proteins compared to the actual mutations [26]. In our study, the amount of abnormalities in Ca²⁺ transients was significantly increased only in the hiPSC-derived CMs with the TPM1-Asp175Asn mutation. Indeed, the amount of irregularities in Ca²⁺ handling properties was similar in the MYBPC3-Gln1061X compared to that in the WT-CMs. The phosphorylation of the proteins was not analyzed in this current study. However, we analyzed the expression of genes related to Ca²⁺ handling and they were at the highest level in hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation. This might be at least partly due to the larger cell size of these CMs. Han and coworkers found decreased level of RYR2 expression in HCM-CMs carrying MYH7-R442G mutation [13], while in our study the expression of RYR2 was significantly higher in both mutations and almost six times higher in the hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation than in the WT-CMs. These observations suggest that abnormal Ca²⁺ transients in HCM-CMs carrying different mutations may be caused by distinct mechanisms.

One of the fundamental features of the HCM is its association with ventricular arrhythmias responsible for severe cardiac malfunctions including sudden cardiac death [27, 28]. We found increased amount of arrhythmogenic events (DADs and EADs) in both HCM-CMs. Furthermore, decreased beating rate was observed in both types of HCM-CMs, which could be due to higher occurrence of DADs between the two consecutive APs. In addition, the APD₉₀ of hiPSC-derived CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X mutation was longer than in WT-CMs, which is in line with previous findings with different mutations [12, 13, 29]. The mechanism of arrhythmias in HCM is not yet fully understood; however, imbalances in Ca²⁺ homeostasis are considered as a main cause of arrhythmias shown in the previous studies [12, 13]. Clinically, patients carrying TPM1-Asp175Asn mutation have been reported to
Figure 5: Cardiac-specific protein expression in hiPSC-derived CMs. (a) Representative images of hiPSC-derived CMs carrying TPM1-Asp175Asn mutation (HCMT) or MYBPC3-Gln1061X mutation (HCMM) and hiPSC-derived control CMs (WT) stained with cTnT, MYBPC, and TPM1. These images are not to quantify the protein expression but to demonstrate the presence of cTnT, MYBPC, and TPM1 proteins in the hiPSC-derived CMs. (b) hiPSC-derived CMs from all cell lines expressed MYBPC, cTnT, and TPM1 proteins. The truncated MYBPC was not detected in HCMM cells (size of the wild type protein 141 kDa and the predicted size of the truncated protein 117 kDa). (c) The expression of MYBPC, cTnT, and TPM1 in WT-CMs, HCMT-CMs, and HCMM-CMs normalized to the expression of β-actin. Protein expressions were quantified from western blots using ImageJ software. Quantitation data show the averages of MYBPC/β-actin, cTnT/β-actin, and TPM1/β-actin relations from hiPSC-derived CMs from two different hiPSC lines in each group. Because of the lack of replicates, statistical analysis was not performed.
be more prone to arrhythmias than those carrying MYBPC3-Gln1061X mutation [30]. Our data with hiPSC-derived CMs support this finding by demonstrating more abnormal Ca\textsuperscript{2+} transients and longer APD\textsubscript{90} in TPM1-Asp175Asn CMs than in HCM-CMs carrying the MYBPC3-Gln1061X mutation.

Like most of the HCM mutations located in the MYBPC3, also MYBPC3-Gln1061X is a nonsense mutation that leads to premature stop-codon [31]. Nonsense mutations are suggested to act through haploinsufficiency in which the mutated protein is either degraded or not produced at all. The truncated form of MYBPC has not been found in human cardiac samples while the total expression level of MYBPC has been reported to vary from being decreased to even increased [32–36]. Interestingly, when studying myectomy samples from HCM hearts with MYBPC3 mutation, Helms et al. observed that the total amount of MYBPC mRNA was increased, while the total amount of MYBPC protein was on the same level compared to that in the control hearts. They hypothesized that the upregulation of the MYBPC compensates the degraded truncated MYBPC protein [35]. We could not detect mutant allele on mRNA expression level or truncated MYBPC protein in hiPSC-derived CMs carrying the MYBPC3-Gln1061X mutation. Similar absence of truncated protein has been reported earlier with a different MYBPC mutation in hiPSC-derived CMs [14]. These data suggest that the mutant mRNA might be degraded. However, further research is still needed to confirm the results and to discover the actual degradation mechanism.

The two HCM mutations analyzed in this study are the most frequent mutations in Finland accounting for about 18% of all Finnish HCM patients [3]. In our study, we found differences in the morphological and biochemical properties, as well as in Ca\textsuperscript{2+} cycling and electrophysiological properties between the CMs carrying either TPM1-Asp175Asn or MYBPC3-Gln1061X mutation. However, we have not analyzed the possible effects of other gene mutations and epigenetic factors on the phenotype differences observed in our study. Additionally, we used only two hiPSC lines from two different patients in each mutation in our experiments. In the future, we need to extend our studies including further patients and studying the effects of additional gene mutations and epigenetic factors. The immature nature of hiPSC-derived CMs is a general limitation when using these cells in disease modeling. Further development in the differentiation and maturation protocols will increase the reliability of studies conducted with hiPSC-derived CMs. Finally, the exact pathophysiology in abnormal Ca\textsuperscript{2+} transients or electrical abnormalities is not known and further research with combined patch clamp and Ca\textsuperscript{2+} imaging is required in the future to reveal the significance of the cellular findings for clinical phenotypes as well as for treatment options.

5. Conclusions

In conclusion, both HCM hiPSC-derived CMs either carrying the TPM1-Asp175Asn or MYBPC3-Gln1061X mutation exhibited pathological changes related to HCM. However, significant differences between the two mutations were observed. The hiPSC-derived cell models, established in this study, can be exploited to study further the pathophysiological mechanisms of HCM as well as to screen drugs and potentially optimize treatments in mutation-specific way.

### Abbreviations

- **AP**: Action potential
- **APA**: Action potential amplitude
- **APD**: Action potential duration
- **bFGF**: Basic fibroblast growth factor
- **BPM**: Beats per minute
- **CM**: Cardiomyocyte
- **CPVT**: Catecholaminergic polymorphic ventricular tachycardia
- **cTnT**: Troponin T
- **DAD**: Delayed after depolarization
- **DAPI**: 40,6-Diamidino-2-phenylindole
- **DCM**: Dilated cardiomyopathy
- **EAD**: Early after depolarization
- **EB**: Embryoid body
- **END-2**: Mouse endodermal-like cells
- **FBS**: Fetal bovine serum
- **HCM**: Hypertrophic cardiomyopathy
- **HCM-CM**: Cardiomyocytes derived from hiPSCs carrying HCM mutation
- **HCMM-CM**: Cardiomyocytes derived from hiPSCs carrying MYBPC3-Gln1061X mutation
- **HCMT-CM**: Cardiomyocytes derived from hiPSCs carrying TPM1-Asp175Asn mutation
- **hiPSC**: Human induced pluripotent stem cell
- **hiPSC-CM**: Cardiomyocytes derived from hiPSCs
- **HPC**: Human pluripotent stem cell
- **HRP**: Horseradish peroxidase
- **ICD**: Implantable cardioverter defibrillator
- **ICD-AS**: Knockout serum replacement
- **ko**: Knockout
- **MDP**: Maximum diastolic potential
- **MEF**: Mouse embryonic fibroblast
- **MYBPC**: Myosin-binding protein C
- **MYH7**: Myosin-β heavy chain
- **NEAA**: Nonessential amino acids
- **RT-STA**: Reverse transcription-specific target amplification
- **SCD**: Sudden cardiac death
- **SEM**: Standard error of the mean
- **TPM1**: -tropomyosin
- **WT-CM**: Cardiomyocytes derived from control hiPSCs

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References


Divergent effects of adrenaline in human induced pluripotent stem cell-derived cardiomyocytes obtained from hypertrophic cardiomyopathy

Chandra Prajapati¹, Marisa Ojala¹ and Katriina Aalto-Setälä¹,²,³,*

ABSTRACT

Hypertrophic cardiomyopathy (HCM) is a common inherited cardiac disease that affects the heart muscle with diverse clinical outcomes. HCM can cause sudden cardiac death (SCD) during or immediately after mild to rigorous physical activity in young patients. However, the mechanism causing SCD as a result of exercise remains unknown, but exercise-induced ventricular arrhythmias are thought to be responsible for this fatal consequence. To understand the disease mechanism behind HCM in a better way, we generated patient-specific induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) from HCM patients carrying either the MYBPC3-Gln1061X or TPM1-Asp175Asn mutation. We extensively investigated the effects of low to high concentrations of adrenaline on action potential characteristics, and the occurrence of arrhythmias in the presence of various concentrations of adrenaline in and washout condition. We classified and quantified different types of arrhythmias observed in hiPSC-CMs, and found that the occurrence of arrhythmias was dependent on concentrations of adrenaline and positions of mutations in genes causing HCM. In addition, we observed ventricular tachycardia types of arrhythmias in hiPSC-CMs carrying the TPM1-Asp175Asn mutation. We additionally examined the antiarrhythmic potency of bisoprolol in HCM-specific hiPSC-CMs. However, bisoprolol could not reduce the occurrence of arrhythmias during administration or during the wash-out condition of adrenaline in HCM-specific hiPSC-CMs. Our study demonstrates hiPSC-CMs as a promising tool for studying HCM. The experimental design used in this study could be suitable and beneficial for studying other components and drugs related to cardiac disease in general.

KEY WORDS: HCM, Arrhythmia, hiPSC-CMs, Adrenaline, Bisoprolol

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a common genetic cardiac disease found worldwide, irrespective of age, sex or ethnic group. HCM is characterized by unexplained left ventricular hypertrophy, mostly in the interventricular septum, but it can also affect other areas of the left ventricle (Maron and Maron, 2013). Histologically, HCM is characterized by myocardial disarray and fibrosis. More than 1400 distinct mutations in >11 genes encoding proteins of cardiac sarcomeres have been associated with HCM, most of which are unique to individual families (Maron and Maron, 2013). Two founder mutations in alpha-tropomyosin (TPM1-Asp175Asn, 6.5%) and in myosin-binding protein C (MYBPC3-Gln1061X, 11.4%) genes together account for ~18% of HCM in Finland (Jääskeläinen et al., 2013). HCM patients often have ventricular arrhythmias such as non-sustained ventricular tachycardia (NSVT) (Monserat et al., 2003) and supraventricular arrhythmias such as atrial fibrillation (Adabag et al., 2005). In worst cases, the first manifestation of HCM could be sudden cardiac death (SCD), usually caused by ventricular tachyarrhythmias (Maron and Maron, 2014). It is known that HCM undergoes remodeling of different ion channels, but how these adaptations endorse repolarization abnormalities and engender lethal arrhythmias is not yet understood (Tomaselli and Marbán, 1999). The reduced number of β-receptor binding sites (Choudhury et al., 1996) and the blunt β-adrenergic signaling pathway have been identified, but interestingly the plasma catecholamine concentration remains unaltered in HCM patients (Schumacher et al., 1995). An earlier study showed that plasma adrenaline does not significantly differ in resting conditions and during exercise between control and HCM groups (Omodani et al., 1998). However, SCD occurs during or immediately after moderate to severe physical activity in HCM patients (van Rijsingen et al., 2011). Although exercise-induced arrhythmias have been associated with an increased risk of SCD, the precise mechanism for SCD has not yet been identified (Gimeno et al., 2009). HCM patients are recommended not to participate in competitive exercise because of impaired hemodynamics and tolerance during exercise (Elliott et al., 2014). Clinically, study of exercise-induced arrhythmias in HCM is challenging. In an early study, eight of 15 HCM patients stopped exercise because of shortness of breath or leg fatigue (Omodani et al., 1998). Currently, there is no disease-specific pharmacological treatment available for HCM patients (Spoladore et al., 2012); an implantable cardioverter-defibrillator (ICD) is the only effective available tool for prevention of SCD (Maron et al., 2000). Therefore, a better understanding of the triggers for lethal ventricular arrhythmias in HCM is necessary.

Prior studies of cardiovascular diseases in animal models have some fundamental problems, such as differences in cardiac physiology, drug responses (O’Hara and Rudy, 2012), and the expression of contractile proteins. Therefore, it is complicated to extrapolate the physiological and pharmacological results from animals to humans (Jung and Bernstein, 2014). In addition, cardiac biopsies from humans are limited and typically obtained from the end stages of cardiac diseases (Barajas-Martinez et al., 2013); hence, it is not possible to understand the mechanisms leading to cardiac diseases. These impedances of in vitro cardiac disease modeling are mostly overcome by the groundbreaking discovery of...
reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007), which can be differentiated into any cell type of the human organism, such as human iPSC-derived cardiomyocytes (hiPSC-CMs) (Kujala et al., 2012; Ojala et al., 2016). In addition, hiPSC-CMs offer a robust platform for in vitro studies of genetic cardiac disorders. We have previously shown that HCM-specific hiPSC-CMs carrying TPM1-Asp175Asn or MYBPC3-Gln1061X mutations showed differences in cell morphology, calcium handling properties and electrophysiological properties, not only from the control but also between these two mutations (Ojala et al., 2016). This study is a follow-up study with comprehensive analysis of the effects of different concentrations of adrenaline on action potential (AP) characteristics and the occurrence of arrhythmias in HCM-specific hiPSC-CMs during and immediately after adrenaline administration. We concentrated on the use of adrenaline because it is a natural physiological agent and plasma adrenaline increases during exercise (Omodani et al., 1998; Warren et al., 1984). Furthermore, we evaluated the beat rate and repolarization variabilities of these hiPSC-CMs to identify the potential link with the occurrence of arrhythmias. Finally, we also examined the efficacy of the β-blocker bisoprolol in subsiding arrhythmias in HCM-specific hiPSC-CMs.

**RESULTS**

**Electrophysiological categorization of hiPSC-CMs**

In this study, hiPSC-CMs were categorized as ventricular-like [action potential duration (APD) at 90%/50% repolarization (APD90/APD50)<1.3] and atrial-like (APD90/APD50>1.35) mainly based on the triangularity of the AP profile.

**Atrial-like hiPSC-CMs**

The minority (~20%) of the hiPSC-CMs exhibited the atrial type of AP profile in hiPSC-CMs derived from control hiPSCs (WT-CMs), hiPSC-CMs carrying the TPM1-Asp175Asn mutation (HCMT-CMs) and hiPSC-CMs carrying the MYBPC3-Gln1061X mutation (HCMM-CMs). Only baseline characteristics were evaluated from atrial-like hiPSC-CMs and none of the AP characteristics of atrial cells were significantly different among WT-CMs (n=21), HCMT-CMs (n=30) and HCMM-CMs (n=34) (Table S1).

**Ventricular-like hiPSC-CMs**

The majority (~80%) of the hiPSC-CMs exhibited the ventricular-like hiPSC-CMs in all groups. The baseline characteristics of ventricular-like hiPSC-CMs have been presented in our previous study (Ojala et al., 2016). In this study, adrenaline and bisoprolol testing was performed only in ventricular-like hiPSC-CMs.

**Voltage-gated ionic currents in hiPSC-CMs**

Previous studies have shown that either gain or loss of function of different voltage-gated ion channels alter the AP profile, and thus facilitate the occurrence of arrhythmias in HCM (Coppini et al., 2013; Gomez et al., 1997). To understand the remodeling of ion channels in HCM, calcium current (\(I_{Ca}\)), transient outward potassium current (\(I_{to}\)) and inward rectifier potassium current (\(I_{K1}\)) were measured in hiPSC-CMs (Fig. 1). The \(I_{Ca}\) current densities were significantly higher in HCMT-CMs (n=23) and HCMM-CMs (n=14) than in WT-CMs (n=15) (P<0.05, WT versus HCMT, from −10 mV to 70 mV, and WT versus HCMM, from 10 mV to 60 mV; one-way ANOVA, post hoc Tukey test). However, no statistical differences in \(I_{Ca}\) current densities were found between HCMT-CMs and HCMM-CMs at any potential (Fig. 1A). By contrast, \(I_{K1}\) current densities were significantly lower in both HCMT-CMs (n=22) and HCMM-CMs (n=23) compared to WT-CMs (n=16) from 20 mV to 70 mV (P<0.05, one-way ANOVA, post hoc Tukey test; Fig. 1B). No significant differences in \(I_{to}\) current densities were found between HCMT-CMs and WT-CMs versus HCMM-CMs versus HCMM-CMs; one-way ANOVA, post hoc Tukey test). Data are mean±s.e.m. Values inside parentheses represent the number of CMs used.

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**Fig. 1. Voltage-gated ionic currents in ventricle-like WT-CMs, HCMT-CMs and HCMM-CMs.**

(A) Current-voltage (I-V) relationship of L-type calcium current (\(I_{Ca}\)) (above) and voltage clamp protocol used (below). \(P<0.05\) from −10 mV to 60 mV (WT-CMs versus HCMT-CMs, one-way ANOVA, post hoc Tukey test), \(P<0.05\) from 10 mV to 80 mV (WT-CMs versus HCMM-CMs, one-way ANOVA, post hoc Tukey test). (B) I-V relationship of transient outward potassium current (\(I_{to}\)) (above) and voltage clamp protocol used (below). \(P<0.05\) from 30 mV to 70 mV (WT-CMs versus HCMT-CMs and WT-CMs versus HCMM-CMs; one-way ANOVA, post hoc Tukey test). (C) I-V relationship of inward rectifier outward current (\(I_{K1}\)) (above) and voltage clamp protocol used (below) (ns at all test potentials, WT-CMs versus HCMT-CMs and WT-CMs versus HCMM-CMs; one-way ANOVA, post hoc Tukey test). Data are mean±s.e.m. Values inside parentheses represent the number of CMs used.
HCMM-CMs at any potential. The $I_{C}\text{-current}$ densities were not significantly different at all potentials tested among groups [nonsignificant (ns), WT-CMs, $n=12$; HCMT-CMs, $n=19$; HCMM-CMs, $n=21$; one-way ANOVA, post hoc Tukey test; Fig. 1C].

**hiPSC-CMs exhibit various types of arrhythmias**

Life-threatening arrhythmias and exercise-induced arrhythmias primarily commence in the ventricle (Monserrat et al., 2003; Gimeno et al., 2009). Thus, we especially focused on ventricular-like hiPSC-CMs to understand these abnormalities. Patch-clamp recordings of self-beating hiPSC-CMs demonstrated various types of arrhythmias (Fig. 2; Fig. S1). Therefore, we categorized and quantified these arrhythmias separately. The arrhythmias were present either exclusively or in the presence of another type of arrhythmia. The most common type of arrhythmia recorded from hiPSC-CMs was delayed afterdepolarization (DAD). DAD was defined as the presence of low-amplitude abnormal depolarization after successive APs that can cause cessation of next spontaneous APs (Fig. 2Aa; Fig. S1A). Both HCMT-CMs (52%, 136/263) and HCMM-CMs (61%, 96/158) exhibited higher occurrence of DADs compared to WT-CMs (23%, 23/102) at baseline (Fig. 2Ad). DADs were also recorded from the atrial hiPSC-CMs (Fig. S2A) in WT-CMs (5%, 1/21), HCMT-CMs (43%, 13/30) and HCMM-CMs (18%, 6/34). The second type of arrhythmia recorded from hiPSC-CMs was phase 3 early afterdepolarization (EAD). Phase 3 was defined as the initiation of the next AP during the third phase of repolarization, i.e. phase 3 in preceding APs (Fig. 2Ba; Fig. S1B). EAD-induced triggered activity in hiPSC-CMs displayed take-off potentials ranging between $-55$ mV and $-65$ mV. When the AP comprising phase 3 EAD was closely analyzed, two distinctive patterns of upstroke velocity were observed. Either the upstroke velocity of AP constituting phase 3 EAD increased compared to its previous AP, or it remained the same (Fig. 2Bb,c). In the baseline condition, the percentage of cells exhibiting phase 3 EAD was higher in both HCMT-CMs (20%, 52/263) and HCMM-CMs (13%, 21/158) than in WT-CMs (3%, 3/102) (Fig. 2Bd). Phase 3 EADs were also recorded from the atrial hiPSC-CMs (Fig. S2B) from WT-CMs (5%, 1/21), HCMT-CMs (13%, 4/30) and HCMM-CMs (3%, 1/34). The third type of arrhythmia exhibited in hiPSC-CMs was burst EAD. This type of EAD was defined as the sudden increase in beating rate triggered by phase 3 EAD (Fig. 2Ca; Fig. S1C). The main characteristics of burst EAD are a faster beat rate and continuous change (either increasing or decreasing) in action potential amplitude (APA). Similar to phase 3 EAD, another notable characteristic observed in some of the burst was an increase
in the upstroke velocity in AP comprising burst EAD (Fig. 2Ch,c). The length and beat rate of the burst EAD observed in hiPSC-CMs ranged from 0.3 s to 27 s and from 50 beats per minute (BPM) to 260 BPM, respectively. Similarly, the take-off potential for burst in our study ranged from −70 mV to −30 mV. Furthermore, the occurrence of burst was two times higher in HCMT-CMs (8%, 20/263) than in HCMM-CMs (4%, 7/185) and WT-CMs (4%, 4/102) in baseline conditions (Fig. 2Cd). Similarly, bursts were also observed in atrial-like hiPSC-CMs (Fig. S2C) from HCMT-CMs (3%, 1/30) and HCMM-CMs (3%, 1/34), but not in those from WT-CMs (0%, 0/21). The fourth type of arrhythmia recorded from hiPSC-CMs was quasi-equilibrium state (QES) EAD (QES-EAD), which was defined as a sudden decrease in APA leading to temporary non-beating membrane potential or oscillation and self-recovery to spontaneous APs (Fig. 2Da; Fig. S1D). As in phase 3 EAD and burst EAD, an increase in upstroke velocity in AP comprising QES-EAD was observed in some cases (Fig. 2Db,c). The take-off potential and duration of QES-EAD recorded in our study varied from −55 mV to −6.5 mV and from 1.4 s to 22.3 s, respectively. At baseline conditions, QES-EADs were observed in WT-CMs (1%, 1/102), HCMT-CMs (1%, 3/263) and HCMM-CMs (1%, 2/158) (Fig. 2Dd). However, QES-EADs were not observed in atrial-like hiPSC-CMs. The fifth type of arrhythmia observed in hiPSC-CMs was ventricular tachycardia (VT), which was defined as phase 3 EAD mediating triggered activity followed by an increased beating rate (Fig. 2Ea; Fig. 3). The main characteristics of VT-EAD were a steady APD and maximum diastolic potential (MDP) during triggered activity. Our results showed that the beat rate and MDP of VT ranged from 70 BPM to 254 BPM and from −69 mV to −45 mV, respectively. Furthermore, another distinguished feature of VT was the possibility of progressively extended triggered activity. Therefore, VTs were subdivided by duration as non-sustained (NSVT, duration <30 s, self-terminated; Fig. 3A), sustained (SVT, duration>30 s, self-terminated; Fig. 3C) and non-recovered (NRVT, not recovered; Fig. 3B). Unlike other types of EAD, an alternation in upstroke velocity constituting VT was not observed. The first four types of arrhythmia (DAD, phase 3 EAD, burst and QES-EAD) were observed in WT-CMs, HCMT-CMs and HCMM-CMs. Interestingly, VTs were only found in HCMT-CMs. To confirm that VTs were exclusively present in HCMT-CMs, we decided to include the clonal line of UTA.02912.HCMT with the TPM1-Asp175Asn mutation called UTA.02913.HCMT (Table S2, Fig. S3). In current clamp recording, we were also able to record VTs in hiPSC-CMs derived from the UTA.02913.HCMT line and therefore confirmed that VT was present in HCMT-CMs.

Baseline variabilities in hiPSC-CMs

Clinically, it is well known that heart rate variabilities and/or ventricular repolarization variabilities differ between control individuals and diseased patients, and are associated with arrhythmias present in the patients (Counihan et al., 1993). In addition, an earlier study showed that hiPSC-CMs exhibited beat rate variability (Mandel et al., 2012). Thus, the beat rate variabilities [standard deviation of instantaneous variability (SD1), standard deviation of long-term variability (SD2), standard deviation of differences between adjacent RR intervals (SDSD) and standard deviation of all RR intervals (SDRR)] and repolarization variabilities [beat-to-beat variability at 50% or 90% repolarization (STV-APD50 or STV-APD90)] were measured to understand whether these variabilities differ in WT-CMs, HCMT-CMs and HCMM-CMs. Thus, recordings of hiPSC-CMs with ≥30 consecutive APs without the presence of any type of arrhythmia were selected from WT-CMs (n=79), HCMT-CMs (n=174) and HCMM-CMs (n=118). First, the three-dimensional plots among SD1, beat rate and APD90, and STV-APD90, beat rate and APD90, were plotted (Fig. S4). After that, correlation tests were performed among beat rate, beat rate and repolarization variabilities, and APDs. The results showed a negative correlation between beat rate and beat rate variabilities in all groups, i.e. the higher the beat rate, the lower the beat rate variability (Fig. S5A-F). By contrast, we found a positive correlation between APDs and APD variability in
all groups, i.e. the longer the APD, the higher the STV (Fig. S5G-L). On the other hand, the results of correlation tests between beat rate variabilities and repolarization variabilities showed a positive relationship between SD1 and APD90 in HCMT-CMs and HCMM-CMs (P<0.05, Pearson’s correlation) but not in WT-CMs (Fig. S6). Therefore, to minimize the effects of beat rate and APDs in their respective variabilities to compare among groups, variabilities data from CMs were again filtered. The newly selected CMs had beat rates ranging from 30 BPM to 60 BPM, and also APDs of 200-600 ms. Consequently, the mean beat rates (WT-CMs 52.5±1.4 versus HCMT-CMs 52.1±1.0 versus HCMM-CMs 55.8±1.3), APD50 (WT-CMs 282.1±8.3 versus HCMT-CMs 283.3±6.7 versus HCMM-CMs 278.9±8.9) and APD90 (WT-CMs 333.6±9.0 versus HCMT-CMs 334.9±7.4 versus HCMM-CMs 328.2±9.6) were not significantly different among the groups (ns, one-way ANOVA, post hoc Tukey test; WT-CMs n=63, HCMT-CMs n=154 and HCMM-CMs n=119; Fig. S7). Thus, we compared variabilities among groups. Fig. 4 shows the variabilities parameters in WT-CMs, HCMT-CMs and HCMM-CMs. All the beat rate variabilities parameters, such as SD1, SD2, SDRR and SDSD, were significantly higher (P<0.05, one-way ANOVA, post hoc ANOVA) in HCMT-CMs and HCMM-CMs than in WT-CMs (Fig. 4A-F). These results imply that both HCMT-CMs and HCMM-CMs exhibit higher degrees of beat variability than WT-CMs. However, STV-APD90 and STV-APD50 were only significantly higher (P<0.05, one-way ANOVA, post hoc Tukey test) in HCMT-CMs compared to WT-CMs (Fig. 4E,F). The CMs exhibiting a higher degree of beat rate and repolarization variabilities were more scattered in Poincaré plot (Fig. 4I and Fig. 3L). In addition, beat rate variabilities and repolarization variabilities of atrial-like hiPSC-CMs from WT-CMs (n=21), HCMT-CMs (n=27) and HCMM-CMs (n=33) were measured, but no significant differences (ns, one-way ANOVA, post hoc Tukey test) in any parameters were found among groups (Table S3).

Adrenaline testing in hiPSC-CMs
Adrenaline levels increase during exercise (Warren et al., 1984), and HCM patients and healthy individuals have similar plasma adrenaline levels during rest and exercise conditions (Omodani et al., 1998). However, HCM patients experience lethal arrhythmia during or immediately after exercise (Gimeno et al., 2009). To understand whether adrenaline causes any adverse effects, hiPSC-CMs were exposed to different concentrations of adrenaline, including 0.1 nM, 0.5 nM, 1 nM, 10 nM, 100 nM, 1 µM and 10 µM. The hiPSC-CMs were administrated only one concentration at a time to avoid the possible effect of desensitization of the receptor (Priori and Corr, 1990). The experimental protocol for the adrenaline testing is shown in Fig. S8.

Effects on AP characteristics during the administration of adrenaline
The effects of adrenaline were calculated as the percentage change compared to pre-adrenaline administration. Fig. 5 summarizes the
effects of various concentrations of adrenaline on AP characteristics in WT-CMs, HCMT-CMs and HCMM-CMs. The beat rates were significantly increased ($P<0.05$, paired t-test), but APD50 and APD90 were significantly decreased ($P<0.05$, paired t-test), from their pre-adrenaline values in a concentration-dependent manner in WT-CMs (Table S4), HCMT-CMs (Table S5) and HCMM-CMs (Table S6) (Fig. 5A-C). Interestingly, APD50 and APD90 were similarly reduced by adrenaline at all concentrations of adrenaline ($P<0.0001$, APD50 versus APD90, Pearson’s correlation; data not shown). Notably, upstroke velocity (dV/dt) was not significantly changed by any concentration of adrenaline in WT-CMs, HCMT-CMs and HCMM-CMs ($P<0.05$, paired t-test; Tables S4-S6). The percentage changes in beat rate and APD90 were not always dependent on their pre-adrenaline values or on one another, as shown by correlation tests (Table S7).

**Effects of adrenaline, during and immediately after administration, on the occurrence of arrhythmias**

To study the effects of adrenaline on arrhythmia frequencies, we quantified those arrhythmias before exposure to adrenaline (baseline condition), during the administration of adrenaline, and immediately after washing off the adrenaline. Table S8 shows the DAD rate in three different conditions in WT-CMs, HCMT-CMs and HCMM-CMs. No significant differences in DAD rate when comparing before, during and immediately after the wash-out condition of different concentrations of adrenaline were observed in WT-CMs, HCMT-CMs and HCMM-CMs ($n$s, Friedman, post hoc Dunn test) at any concentration tested (Table S8). Similarly, phase 3 EAD rate at different concentrations were also quantified, but significant changes were not observed at any concentration in any group ($n$s, Friedman, post hoc Dunn test; Table S9). At higher concentrations of adrenaline (>1 nM), bursts were not observed in WT-CMs or HCMM-CM in the presence of adrenaline (Table S10). However, bursts were recorded at all concentrations of adrenaline tested both during administration and after washing out adrenaline in HCMT-CMs (Table S10). Similarly, QES-EADs were observed during the administration of adrenaline, except at the 100 nM concentration in HCMT-CMs (Table S11). By contrast, QES-EADs were observed in all three conditions at 100 nM adrenaline in WT-CMs (Table S11). Furthermore, when studying the occurrence of different types of VTs in HCMT-CMs, only NSVTs were observed at baseline conditions. However, transition from NSVT to sustained...
SVT and/or NRVT was observed during the administration and wash-out condition of adrenaline (Fig. 3; Table S12). NSVTs were observed during the wash-out conditions from the 100 nM and 1 µM concentrations of adrenaline (Table S12).

Effects on variabilities during the administration of adrenaline
We also quantified the beat rate variabilities and repolarization variabilities in hiPSC-CMs after the administration of adrenaline. Similarly, as before, the percentage changes in variabilities were calculated with respect to pre-adrenaline administration. Fig. 6A-D and Fig. 6E,F summarize the changes in beat rate variabilities and repolarization variabilities, respectively, with various concentrations of adrenaline. There were no major significant differences in beat rate variabilities in WT-CMs, HCMT-CMs and HCMM-CMs at any adrenaline concentration (ns, one-way ANOVA, post hoc Tukey test). Only the percentage changes in both repolarization variabilities were significantly less at 1 nM and 10 nM of adrenaline in HCMT-CMs when compared to HCMM-CMs or WT-CMs (*P<0.05, WT-CMs versus HCMT-CMs or HCMM-CMs; #P<0.05, HCMT-CMs versus HCMM-CMs; one-way ANOVA, post hoc Tukey test. Table shows the number of cells used in each group at different adrenaline concentrations. (For more detail, see Tables S13-15.)

Pharmacological treatment of HCM-specific hiPSC-CMs
Although there is currently no HCM-specific drug available, β-blockers (class-II antiarrhythmic drug) are commonly prescribed to HCM patients (Spoladore et al., 2012). In this study, bisoprolol was used to investigate whether it would exert antiarrhythmic effects in our hiPSC-CMs. Detailed information about the experimental design for bisoprolol treatment is shown in Fig. S9. In brief, extracellular solution with adrenaline was used prior to the combination of adrenaline and bisoprolol, followed by solution with bisoprolol alone.

Role of bisoprolol in HCMT-CMs
To examine the efficacy of β-blockers in HCMT-CMs, we chose to administer 0.5 nM adrenaline because all types of arrhythmias were observed at this concentration. At first, we evaluated the potency of 1 µM bisoprolol for 0.5 nM adrenaline in HCMT-CMs. DAD and phase 3 EAD rates were not significantly (ns, Kruskal–Wallis, post hoc Dunn test) reduced by bisoprolol in both the presence and wash-out condition of 0.5 nM adrenaline (Fig. 7A,B,J,K). Bursts were reduced by 1 µM bisoprolol in the presence of 0.5 nM adrenaline (Fig. 7C) but not during the wash-out condition of 0.5 nM adrenaline (Fig. 7L). In addition, 1 µM bisoprolol could not reduce the occurrence of QES-EAD in the presence or in the wash-out condition of 0.5 nM adrenaline (Fig. 7D,M). Next, the 10 µM bisoprolol concentration was tested to determine whether a higher

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**Fig. 6. Superimposed traces representing the effects of different concentrations of adrenaline on beat rate and repolarization variabilities.**

(A-F) Percentage change in SD1 (A), SD2 (B), SDRR (C), SDSD (D), STV-APD50 (E) and STV-APD90 (F) with respect to pre-adrenaline administration condition in WT-CMs, HCMT-CMs and HCMM-CMs. Data are means±s.e.m. *P<0.05, WT-CMs versus HCMT-CMs or HCMM-CMs; #P<0.05, HCMT-CMs versus HCMM-CMs; one-way ANOVA, post hoc Tukey test. Table shows the number of cells used in each group at different adrenaline concentrations. (For more detail, see Tables S13-15.)
concentration of bisoprolol could reduce the arrhythmias. However, 10 µM bisoprolol could not decrease the DAD and phase 3 EAD rates significantly (ns, Kruskal–Wallis, post hoc Dunn test), or reduce the occurrence of burst and QES-EAD, in both the presence and wash-out condition of 0.5 nM adrenaline (Fig. 7A-D,J-M). In the presence of either 1 µM or 10 µM bisoprolol, VTs were observed, indicating that bisoprolol failed to prevent the initiation and termination of lethal arrhythmias in HCMT-CMs (Fig. 7E,N). The role of bisoprolol in beat rate variabilities and repolarization variabilities were studied, but no significant changes (ns, one-way ANOVA, post hoc Tukey test) were found in the presence of 1 µM or 10 µM bisoprolol (Fig. 7F-I).

**Role of bisoprolol in HCMM-CMs**

We also investigated the potency of both 1 µM and 10 µM bisoprolol in the presence and wash-out conditions of 10 nM adrenaline in HCMM-CMs. As shown above, the higher concentrations of adrenaline decreased the occurrence of arrhythmias in HCMM-CMs; thus, a lower concentration of adrenaline was preferred, and the 10 nM adrenaline concentration was chosen. As in HCMT-CMs, neither 1 µM nor 10 µM bisoprolol reduced the DAD rate and phase 3 EAD rate significantly (ns, Kruskal–Wallis, post hoc Dunn test) in the presence and wash-out condition of 10 nM adrenaline (Fig. 8A,B,I,J). Burst was not observed in the presence of 1 µM bisoprolol and 10 nM adrenaline and during the wash-out condition of 10 nM adrenaline (Fig. 8C,K). However, bursts were observed in the presence of 10 µM bisoprolol and 10 nM adrenaline, but not during the wash-out condition of 10 nM adrenaline (Fig. 8C,K). Notably, QES-EADs were increased by both 1 µM and 10 µM bisoprolol in the presence and wash-out condition of 10 nM adrenaline (Fig. 8D,L). Furthermore, the beat rate variabilities and repolarization variabilities were not significantly changed (ns, one-way ANOVA, post hoc Tukey test) by bisoprolol in HCMM-CMs (Fig. 8E-H).

**DISCUSSION**

In our previous study (Ojala et al., 2016), we demonstrated the phenotypic characteristics of HCM in hiPSC-CMs, such as a higher degree of arrhythmias and abnormal calcium handling at baseline conditions. To our knowledge, this is the first study investigating the electrophysiological properties of HCM-specific hiPSC-CMs during the administration and immediately after the wash-out conditions of various concentrations of adrenaline. Furthermore, we investigated the potency of bisoprolol as an antiarrhythmic drug in HCM-specific hiPSC-CMs. Our main findings in the current study are as follows: (1) $I_{Ca}$ and $I_{To}$ are remodeled in HCM-specific hiPSC-CMs; (2) frequencies of ventricular arrhythmias are dependent on concentrations of...
adrenaline and position of mutation in genes causing HCM; (3) VT types of arrhythmia are observed in HCMT-CMs, but not in HCMM-CMs; and (4) bisoprolol cannot cure arrhythmias in HCM-specific hiPSC-CMs.

Remodeling of ion channels and arrhythmias

The calcium current densities in both HCM-specific hiPSC-CMs were higher than in WT-CMs, which might explain the prolongation of APD90 in HCM-CMs as shown in our previous study (Ojala et al., 2016). However, in our earlier study, APD90s of HCMT-CMs were longer than those of HCMM-CMs, despite similar ICa (Ojala et al., 2016). Although ICa is one of the main components in determining the APD (Han et al., 2014), there are other possible reasons for APD prolongation, such as increased late sodium current (Coppini et al., 2013), decreased rapid-delayed rectifier potassium current (Ma et al., 2015). Interestingly, decreased Ito current densities in our HCM-specific hiPSC-CMs was similar to what was observed in isolated cells from HCM patients (Coppini et al., 2013), but this was contradictory to hiPSC-CMs from HCM patients in another study (Han et al., 2014). Furthermore, we found contradictory results in IK1 current densities from previous publications, in which isolated cells from HCM patients were used (Coppini et al., 2013). The remodeling of ion channels is considered one of the causes of arrhythmias in HCM (Tomaselli and Marbán, 1999; Knollmann et al., 2003). The characteristics of arrhythmias in HCM have been extensively studied using different models, such as experimental (animal and isolated CMs) (Coppini et al., 2013; Knollmann et al., 2003) and computer models (Passini et al., 2016; Zile and Trayanova, 2017). DAD and EAD could coexist and affect one another because of bidirectional calcium-voltage coupling (Song et al., 2015). The potential mechanisms of DAD (January and Fozzard, 1988), phase 3 EAD (Burashnikov and Antzelevitch, 2003), burst (Chang et al., 2012), long plateau (Song et al., 2015) and VT (Maruyama et al., 2011) types of arrhythmia were described in early studies. Importantly, one distinguished feature observed from the first derivative of all EADs was that the upstroke velocity of EAD was much lower compared to that in APs comprising EAD [Fig. 2, first derivative (Bb,Cb,Db) and phase plots (Bc,Cc,Dc)]. Additionally, phase 3 EAD, burst and QES-EAD arrhythmias recorded during the current clamp might correspond to the double peak, oscillation and plateau types of calcium dynamics, respectively, shown in our earlier study (Ojala et al., 2016). Taken together, these results indicate that these arrhythmias are possibly calcium-driven phenomena.
**Variabilities in hiPSC-CMs**

Clinically, the heart rate variabilities in HCM gave mixed results as those parameters depend on age of patients and thickness of left ventricular mass (Counihan et al., 1993; Alter et al., 2006). The variabilities of repolarization indicate a repolarization reserve, suggesting that the higher the variability, the larger the susceptibility for repolarization-dependent ventricular arrhythmias (Varró and Baczkó, 2011). In an early clinical study, the short-term variability (STV) of QT interval was significantly higher in HCM patients than in healthy individuals (Orosz et al., 2015). Another study found that CMs exhibiting ventricular fibrillation (VF) have increased variabilities in APD50 and APD90 than CMs not displaying VF (Sridhar et al., 2008). In our results, VT arrhythmias were only seen in HCMT-CMs, which display significantly higher repolarization variability than WT-CMs. Thus, this indicates the link between repolarization variabilities and VT types of arrhythmias.

**Role of adrenaline in the occurrence of arrhythmias**

Most previous studies have focused on isoproterenol for β-adrenergic stimulation and shown an increased amount of arrhythmias in HCM models (Lan et al., 2013; Han et al., 2014; Knollmann et al., 2003). However, we used adrenaline over isoproterenol for β-adrenergic stimulation in this study because adrenaline is an endogenous catecholamine, whereas isoproterenol is an exogenous agent, i.e. not a natural compound. Although isoproterenol only activates the β-(both β1 and β2) adrenergic pathway, adrenaline stimulates both α- and β-adrenergic receptors (Brodde and Michel, 1999). In human, the physiological plasma adrenaline concentration during rest is ~0.12 nM/l (Omodani et al., 1998; Warren et al., 1984), and adrenaline causes a positive inotropic effect and a decrease in APD90, mainly via the β-adrenergic pathway (Jakob et al., 1988). In contrast to these results, our previous work on catecholaminergic polymorphic ventricular tachycardia (CPVT) using hiPSC-CMs showed that administration of adrenaline (1 µM) increased the arrhythmic episodes in CPVT-specific hiPSC-CMs (Kujala et al., 2012; Penttinen et al., 2015). These conflicting results demonstrate that the different gene mutations inside cardiomyocytes (CMs) give distinct responses, even though the adrenaline levels are the same. Although the TPM1-Asp175Asn mutation causing HCM has been considered benign or to represent intermediary risk (Coviello et al., 1997), Hedman and co-workers suggested that HCM patients carrying TPM1-Asp175Asn mutation were at increased risk of fatal arrhythmias, and a number of these patients had SCD at a young or middle age, or presented two or three clinical markers for increased risk of SCD [family history of SCD, syncpe, NSVT, pathological blood pressure response, marked (>2.5 cm) hypertrophy] (Hedman et al., 2004). On the other hand, the MYBPC3-Gln1061X mutation exhibited age-related penetrance with delayed onset of the disease (Jääskeläinen et al., 2002). Moreover, NSVTs were observed in a higher number of HCM patients carrying MYBPC3-Gln1061X mutations than HCM patients carrying the TPM1-Asp175Asn mutation (23% versus 10%) during 24-h electrocardiogram monitoring (Jääskeläinen et al., 2013). However, in our hiPSC-CM study, we only observed VT types of arrhythmia in HCMT-CMs, thus our finding is in line with earlier clinical studies of patients with the TPM1-Asp175Asn mutation being at increased risk of arrhythmia. In previous clinical exercise tests, some HCM patients experienced NSVT and VF, and all were asymptomatic (Gimeno et al., 2009). In our study, HCM patients whose hiPSC-CMs showed NSVT are also clinically asymptomatic; however, the NSVT transition to SVT or NRVT occurred only after the administration of adrenaline. Xie et al. (2014) presented a possible mechanism of this transition via β-adrenergic stimulation. Although the occurrence of arrhythmias during exercise is infrequent in HCM, existence of such arrhythmias can be linked to increased risk of SCD (Gimeno et al., 2009). Patel et al. (2014) found that the peak heart rate during exercise testing was lower in HCM patients than in controls. The possible reason for slower heart rate might be reduction in β-adrenergic receptor density (Choudhary et al., 1996) and a desensitized cardiac β-adrenergic system under normal plasma catecholamines in HCM (Schumacher et al., 1995).

**Pharmacology in HCM-specific hiPSC-CMs**

Bisoprolol is a selective β1-adrenoceptor blocker (Brixius et al., 2001) and has been used to improve exercise capacity in heart failure (Issa et al., 2007; Dubach et al., 2002). In another clinical study on heart failure, bisoprolol reduced heart rate and increased the heart rate variability parameter (Pouset al., 1996). Contradictory to our results, earlier studies claimed that β-blockers were able to reduce arrhythmias in HCM-specific hiPSC-CMs (Lan et al., 2013; Han et al., 2014). The possible reasons for these conflicting results might be differences in the study design, including long-term administration versus short-term administration, different concentration and different types of β-adrenergic agonist/antagonist used in our study. Furthermore, we specifically categorized the arrhythmias presented in our hiPSC-CMs, which had not been done in earlier studies (Han et al., 2014; Lan et al., 2013). In addition, an early study comparing antiarrhythmic drug therapy and ICD showed that ICD was superior to antiarrhythmic drugs among patients who experienced VF or sustained VT [Antiarrhythmics versus Implantable Defibrillators (AVID) Investigators, 1997]. The previous study showed that programmed ventricular stimulations (PVS) induced SVT or VF in 33% (7/21) of HCM patients carrying the TPM1-Asp175Asn mutation, although 57% (4/7) of those vulnerable HCM patients were under β-blocker therapy (Hedman et al., 2004), indicating that β-blocker treatment is suboptimal in arrhythmia prevention in HCM. Taken together, these results indicate that ICD therapy is the only proven technique for effective termination of VT episode, preventing SCD in high-risk HCM patients (Trivedi and Knight, 2016).

**Study limitations**

Although our comprehensive study revealed previously unknown roles of adrenaline and bisoprolol in HCM-specific hiPSC-CMs, it also raises some new questions. Why do hiPSC-CMs with different HCM-causing mutations respond differently to endogenous adrenaline and why are VT types of arrhythmia exclusively presented in hiPSC-CMs with mutation in TPM1-Asp175Asn? However, it is beyond the scope of this study to suggest a promising mechanism of the arrhythmias in these hiPSC-CMs. In addition, owing to their developmental immaturity, these hiPSC-CMs exhibit some essential differences from adult CMs, such as lack of t-tubules and slow-conducting intercellular communication; thus, microscopic single hiPSC-CMs cannot completely model the whole macroscopic human heart. Moreover,
the mutations associated with HCM patients studied herein are the most common mutations in Finland. Therefore, these results might only be specific to these mutations and cannot readily be extrapolated to all HCM patients. Generating the isogenic control cell lines by correcting the HCM-causing mutation with the help of CRISPR/Cas9 to understand the mechanism of HCM diseases in a better way is the aim of future studies.

Conclusions
This study has clearly shown that while studying β-adrenergic stimulation and blockage in HCM, it is extremely important to address not only during the administration of agonists/antagonist, but also immediately after washing out. Furthermore, the hiPSC-CM model provides a safe and robust platform to study the genetic cardiac diseases. We strongly believe that our results contribute to a better understanding of HCM and create a foundation for future investigation.

MATERIALS AND METHODS
Ethical approval
BioMediTech (Heart Group) has received permission from the Ethics Committee of Pirkkamaa Hospital District to conduct research in hiPSCs (R08070). Skin biopsies for hiPSC establishment were received from the Heart Hospital, Tampere University Hospital, Tampere, Finland. The patients donating skin biopsies provided written informed consent.

Patient-specific hiPSC lines and hiPSC-derived CMs
Seven hiPSC lines were used in this study: three lines with mutation in TPM1 (TPM1-Asp175Asn) (U.TA.13602.HCMT, UTA.02912.HCMT and UTA02913.HCMT), two lines with mutation in MYBPC3 (MYBPC3-Gln1061X) (UTA.06108.HCMM and UTA.07801.HCMM), and two lines derived from healthy individuals (UTA.04602.WT and UTA.04511.WT). The characterization of all other hiPSC lines except UTA.02913.HCMT was performed in our previous study (Ojala et al., 2016). Details on hiPSC lines and individuals from whom hiPSC lines were derived are presented in Table S2. The characterization of UTA.02913.HCMT is shown in Fig. S3. hiPSCs were differentiated into CMs (hiPSC-CMs) by co-culturing with mouse visceral endoderm-like cells (END-2) as described in our previous study (Ojala et al., 2016). In this study, 40- to 85-day-old hiPSC-CMs were used for experiments, and control cell lines and cell lines with each mutation were combined in groups. Two hiPSC-CMs derived from two healthy individuals without any known mutations causing HCM were combined to make the WT-CM group. Furthermore, three hiPSC-CMs from two different individuals carrying the same TPM1 (TPM1-Asp175Asn) mutation were combined to make the HCM-CM group. Similarly, two hiPSC-CMs from two different individuals carrying the same MYBPC3 (MYBPC3-Gln1061X) mutation were combined to make the HCM-CM group.

Chemicals and drugs
All drugs and chemicals for electrophysiology experiments were purchased from Sigma-Aldrich (USA) unless otherwise specified. Potassium methanesulfonate (KMeSO₄) was ordered from MP Biomedicals (USA). (+)-Epinephrine hydrochloride (adrenaline) was dissolved in Milli-Q water, tightly sealed in an Eppendorf tube and stored at −20°C. Cadmium chloride (CdCl₂) and barium chloride (BaCl₂) were dissolved in Milli-Q water and stored at 4°C. On the day of experiments, the drugs were diluted to the final concentration in extracellular solution. 4-Aminopyridine (4-AP) was dissolved in the extracellular solution on the day of experiment. Amphotericin-B was first dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and then added to intracellular solution to a final concentration of 0.24 mg/ml and stored at 4°C during experiments.

Current clamp
APs were recorded in the gap-free mode from hiPSC-CMs using a perforated patch configuration with amphotericin B as previously described (Ojala et al., 2016). In brief, the hiPSC-CMs were continuously perfused at 36±1°C with an extracellular solution containing 143 mM NaCl, 4.8 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose and 10 mM HEPES (pH was adjusted to 7.4 with NaOH). The intracellular solution contained 132 mM KMeSO₄, 20 mM KCl, 1 mM MgCl₂, 4 mM EGTA and 1 mM CaCl₂ (pH was adjusted to 7.2 with KOH).

Voltage clamp
Once the stable baseline APs were obtained, the amplifier was switched into voltage clamp mode to record ionic currents maintaining the same extracellular solution. Ionic currents were divided by cell capacitances and presented as pA/pF. All measurements were performed at 36±1°C. The calcium current (ICa) was measured with a depolarizing potential of −50 mV to −80 mV with 10 mV increments. The holding potential (HP) was −40 mV to inactivate the sodium channels, and 3 mV 4-AP was used in the extracellular solution to block the transient outward potassium current (IK). Early study showed that there was less contamination from sodium ions in the calcium current measurement (Fatima et al., 2013). IK was sodium dependent, and either removing or blocking sodium ions reduced the peak IK current (Dukes and Morad, 1991). Thus, sodium ions were not removed and sodium channels were not blocked in this study. Next, ICa was measured with the two-step protocol from a HP of −80 mV: a first step of −50 mV was used to inactivate the sodium channels and then to test potentials from −50 mV to 70 mV with a step size of 10 mV. We added 300 µM CdCl₂ to the extracellular solution to block the calcium currents. The inward rectifier potassium current (IK₁) was measured with 2 mM BaCl₂-sensitive current in the presence of 300 µM CdCl₂ and 3 mM 4-AP. The currents were elicited by a depolarizing potential of −140 mV to 0 mV with 10 mV increments, and HP was −40 mV. In addition, the IK₁ current was extracted by the subtraction of currents recorded with and without the presence of BaCl₂ in the extracellular solution.

Variability analysis
Beat rate variability (BRV) was assessed from ≥30 consecutive APs without the presence of arrhythmias. SD1 is the standard deviation (SD) of the instantaneous (STV) beat-to-beat interval variability. SD2 is the SD of the SD1, SD1 and SD2 were calculated using the formulae SD1 = 1/2×SDSD2 and SD2 = 2×SDSD2−1/2×SDSD2, respectively, in which SDSD represents the SD of all AR intervals and SDSD represents the SD of differences between adjacent RR intervals. Similarly, the STV-APD50 and STV-APD90 were assessed in the same selected for BRV using the formulae STV-APD50 = ∑[APD₉₀₋₄₋₁ – APD₉₀₋₄₋₁]/[n√2] and STV-APD₉₀ = ∑[APD₉₀₋₄₋₁ – APD₉₀₋₄₋₁]/[n√2], respectively. n represents the number of APs used to calculate variabilities.

Data analysis
Recorded APs were analyzed using custom-made software in Origin9.1 (OriginLab Corp., USA) to extract BPM, APD50 and APD90, APA, dV/dt and MDP. The recorded ionic currents were analyzed using Clampfit 10.7 (Molecular Devices, USA). The SD1, SD2, SDRR, SDSD, STV-APD50 and STV-APD90 were calculated using custom-made software in Matlab R2015a (MathWorks, Inc., USA).

Statistical analysis
For independent group comparisons, a one-way ANOVA was followed by Tukey’s post hoc test was used for normally distributed data. In addition, a paired t-test was used for the dependent normally distributed data. For the three independent variables, the Kruskal–Wallis test followed by the Dunn test was used for non-normally distributed data. For the three dependent variables, the Friedman test followed by the Dunn test was used for non-normally distributed data. A two-tailed P<0.05 was considered statistically significant. Data are presented as mean±standard error of mean (s.e.m.).

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.032896: supplenental

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Antiarhythmics versus Implantable Defibrillators (AVID) Investigators
Supplementary information available online at

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Author contributions
The authors declare no competing or financial interests.

Competing interests

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Biophys. J.
early afterdepolarization-mediated triggered activity in cardiac monolayers. H. S., Tung, L., Marba

107
, 287-300.

Am. Heart J.

143
, 676-683.


## Supplementary Information

### Table S1. Summary of action potential properties of atrial-like hiPSC-CMs.

<table>
<thead>
<tr>
<th></th>
<th>Beats rate (BPM)</th>
<th>APD(_{50}) (ms)</th>
<th>APD(_{90}) (ms)</th>
<th>APA(_{\text{Max}}) (mV)</th>
<th>MDP (mV)</th>
<th>V(_{\text{max}}) (V/S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CMs (21)</td>
<td>87.2 ± 6.9</td>
<td>110.2 ± 13.9</td>
<td>166.0 ± 17.6</td>
<td>110.4 ± 2.5</td>
<td>-74.6 ± 1.3</td>
<td>73.9 ± 10.8</td>
</tr>
<tr>
<td>HCMT-CMs (30)</td>
<td>75.2 ± 4.9</td>
<td>155.6 ± 13.0</td>
<td>225.1 ± 18.3</td>
<td>108.2 ± 2.1</td>
<td>-71.9 ± 1.0</td>
<td>63.3 ± 9.1</td>
</tr>
<tr>
<td>HCMM-CMs (34)</td>
<td>76.6 ± 3.0</td>
<td>141.8 ± 9.6</td>
<td>210.4 ± 14.4</td>
<td>112.1 ± 2.0</td>
<td>-74.0 ± 0.9</td>
<td>65.9 ± 6.9</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. No significant differences were found in any parameters among groups (ns, one-way ANOVA, post hoc Tukey test).
Figure S1. Various types of arrhythmias recorded in ventricular-like hiPSC-CMs. (A) Representative APs with single and multiple DADs (B) Representative APs with different amplitude and APDs of Phase 3 EAD (C) Representative APs with increasing and decreasing APA in burst EAD (D) Representative APs with varying plateau potential in QES-EAD. Dashed line represent 0 mV.
Table S2: Detailed information of hiPSC lines, mutations and individuals from which hiPSC lines were derived.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Reprogramming method</th>
<th>Mutation</th>
<th>Sex</th>
<th>Age</th>
<th>IVS (mm)</th>
<th>Other symptoms</th>
<th>SCD in family</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTA.04602.WT</td>
<td>Retrovirus</td>
<td></td>
<td>F</td>
<td>56</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTA.04511.WT</td>
<td>Sendai virus</td>
<td></td>
<td>M</td>
<td>34</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTA.13602.HCMT</td>
<td>Sendai virus</td>
<td>TPM1-Asp175Asn</td>
<td>F</td>
<td>48</td>
<td>16</td>
<td>Collapsed when 20 years old (normal heart structure)</td>
<td>mother at age of 51</td>
<td>none</td>
</tr>
<tr>
<td>UTA.02912.HCMT</td>
<td>Sendai virus</td>
<td>TPM1-Asp175Asn</td>
<td>M</td>
<td>33</td>
<td>26</td>
<td>Asymptomatic</td>
<td>One family member at age of 21</td>
<td>β-blocker</td>
</tr>
<tr>
<td>UTA.02913.HCMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTA.06108.HCMM</td>
<td>Retrovirus</td>
<td>MYBPC3-Gln1061X</td>
<td>M</td>
<td>55</td>
<td>22</td>
<td>Asymptomatic</td>
<td>father at age of 36 uncle at age of 38</td>
<td>none</td>
</tr>
<tr>
<td>UTA.07801.HCMM</td>
<td>Retrovirus (Cre-LoxP)</td>
<td>MYBPC3-Gln1061X</td>
<td>M</td>
<td>61</td>
<td>25</td>
<td>Atrial fibrillation</td>
<td>none</td>
<td>β-blocker ICD</td>
</tr>
</tbody>
</table>

IVS: Intraventricular septum
SCD: Sudden cardiac death
ICD: Implantable cardioverter defibrillator
Figure S2. Summary of baseline characteristics of atrial-like hiPSC-CMs. Representative APs with presence of DADs (Aa) its first derivative (Ab) its phase plot (Ac) and percentage of cells exhibiting DADs (Ad) Representative APs with phase 3 EAD (Ba) its first derivative (Bb) its phase plot (Bc) and percentage of cells exhibiting Phase 3 EAD (Bd), Representative APs with burst (Ca) its first derivative (Cb) its phase plot (Cc) and percentage of cells exhibiting Burst (Cd). Respective arrhythmias are indicated by an arrow (▼).
Figure S3. Characterization of UTA.02913.HCMT hiPSC line. (A) The hiPSCs formed colonies expressing Oct4, Nanog, Sox2, TRA-1-81, TRA-1-60 and SSEA-4. (B) hiPSCs expressed endogenous SOX2 (151bp), Nanog (287bp), OCT4 (144bp), cMYC (328bp) and REX1 (306bp). Virally transferred Sendai exogenes exo-OCT4 (483bp), exo-KLF4 (410bp), exo-SOX2 (451bp) and exo-cMYC (532bp) were absent in hiPSCs. + indicates positive controls. (C) As a proof of pluripotency, hiPSCs were differentiated into EBs, which expressed markers from all germ layers: endoderm (SOX17 (120bp), AFP (209bp)), mesoderm (α-cardiac actin (486bp), KDR (218bp)) and ectoderm (SOX1 (166bp), PAX6 (274bp)). GAPDH (302bp) was used as a housekeeping control in each PCR experiment. (D) The hiPSC line was karyotypically normal (46, XY) in KaryoLite BoBs Assay (PerkinElmer).
Figure S4: Three-dimensional representation of beat rate and repolarization variabilities and AP parameters. (A-C) 3-D plot of beat rate, APD90 and SD1 and (D-F) beat rate, APD90 and STV-APD90 in WT-CMs (n=79, ■), HCMT-CMs (n=174, ●) and HCMM-CMs (n=118, ▲).
Figure S5. Beat rate and repolarization variabilities parameters as a function of BPM, APD50 and APD90. Correlation between beat rate variabilities with their beat rates and APD variabilities with their APDs in WT-CMs (n=79, ■), HCMT-CMs (n=174, ●) and HCMM-CMs (n=118, ▲). ‘r’ represents the correlation coefficient where ‘−’ sign means inverse relationship and vice versa.
Figure S6. Graph of SD1 vs. APD90. Correlation between SD1 and APD90 in WT-CMs (n=79, ■), HCMT-CMs (n=174, ●) and HCMM-CMs (n=118, ▲). No correlation between SD1 and APD90 was found in WT-CMs.
Figure S7. Comparison of BPM, APD50 and APD90. Summary of hiPSC-CMs fulfilling the conditions of 30 to 60 bpm, and APD of 200 to 600 ms in WT-CMs (n=63), HCMT-CMs (n=154) and HCMM-CMs (n=119). No significant differences were found among the groups. (ns, one-way ANOVA, post hoc Tukey test)
Table S3. Summary of variabilities in atrial-like hiPSC-CMs.

<table>
<thead>
<tr>
<th></th>
<th>SD1</th>
<th>SD2</th>
<th>SDRR</th>
<th>SDSD</th>
<th>SV_ADP90</th>
<th>SV_APD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CMs (21)</td>
<td>29.6±3.9</td>
<td>36.9±6.3</td>
<td>33.7±5.1</td>
<td>41.9±5.5</td>
<td>1.6±0.2</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>HCMT-CMs (27)</td>
<td>29.1±2.9</td>
<td>38.0±3.6</td>
<td>34.1±3.2</td>
<td>41.2±4.2</td>
<td>1.9±0.2</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>HCMM-CMs (33)</td>
<td>27.1±5.5</td>
<td>33.0±4.0</td>
<td>30.4±3.7</td>
<td>38.3±5.0</td>
<td>2.0±0.3</td>
<td>1.7±0.2</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM. No significant differences were found in any parameters among groups (ns, one-way ANOVA, post hoc Tukey test)
Figure S8. Schematic diagram of experimental setup and protocol used for adrenaline testing. Once the stable baseline was recorded with ES, adrenaline with one concentration (ESa) at a time was infused. Adrenaline was passed through the cells for a minimum of 4 min, after which extracellular without adrenaline (ES) was passed through cells to wash out adrenaline from the recording chamber. Stable APs were selected for baseline characteristics. APs after a minimum of 2 min of adrenaline infusion were selected to study the effect of adrenaline. Furthermore, to study the effect of adrenaline in the washing-out condition, maximum up to 300 ms immediately after the adrenaline being washed out were selected for analysis.
Table S4. Percentage change in AP characteristic in WT-CMs at different concentrations of adrenaline.

<table>
<thead>
<tr>
<th>Adrenaline (nM)</th>
<th>Δ%BPM</th>
<th>Δ%APD90</th>
<th>Δ%APD50</th>
<th>Δ%APA</th>
<th>Δ%MDP</th>
<th>Δ%dVdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (n=13)</td>
<td>4.99±1.38</td>
<td>-10.0±1.37</td>
<td>-7.78±1.09</td>
<td>-0.41±0.71</td>
<td>-0.61±0.75</td>
<td>1.04±4.36</td>
</tr>
<tr>
<td>0.5 (n=10)</td>
<td>10.09±1.89</td>
<td>-7.97±0.78</td>
<td>-5.99±0.84</td>
<td>0.15±0.36</td>
<td>-0.43±0.57</td>
<td>11.36±5.06</td>
</tr>
<tr>
<td>1 (n=17)</td>
<td>11.71±1.38</td>
<td>-11.27±1.32</td>
<td>-9.57±1.27</td>
<td>-1.02±0.47</td>
<td>-1.09±0.62</td>
<td>3.67±2.91</td>
</tr>
<tr>
<td>10 (n=8)</td>
<td>40.66±4.2</td>
<td>-17.57±2.53</td>
<td>-17.94±3.08</td>
<td>-1.32±0.69</td>
<td>-1.52±1.25</td>
<td>4.35±6.35</td>
</tr>
<tr>
<td>100 (n=12)</td>
<td>47.67±4.94</td>
<td>-14.53±1.99</td>
<td>-15.30±2.11</td>
<td>-3.29±0.89</td>
<td>-2.91±0.77</td>
<td>-14.72±10.98</td>
</tr>
<tr>
<td>1000 (n=11)</td>
<td>59.60±9.42</td>
<td>-17.38±1.94</td>
<td>-17.46±1.96</td>
<td>-1.61±0.64</td>
<td>-0.54±0.60</td>
<td>-4.42±9.05</td>
</tr>
<tr>
<td>10000 (n=14)</td>
<td>68.67±8.29</td>
<td>-22.44±2.64</td>
<td>-24.47±2.88</td>
<td>-5.51±0.69</td>
<td>-4.31±0.63</td>
<td>0.02±7.28</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. * P < 0.05, ** P < 0.005 and *** P < 0.0001 (paired t-test, pre adrenaline vs. post adrenaline). The ‘–’ sign means that the value had decreased from its corresponding pre-drug administration. n represents the number of cells used in each experiment.
Table S5. Percentage change in AP characteristic in HCMT-CMs at different concentrations of adrenaline.

<table>
<thead>
<tr>
<th>Adrenaline (nM)</th>
<th>Δ% BPM</th>
<th>Δ% APD90</th>
<th>Δ% APD50</th>
<th>Δ% APA</th>
<th>Δ% MDP</th>
<th>Δ% dVdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (n=29)</td>
<td>7.73±1.10***</td>
<td>-6.28±1.11***</td>
<td>-5.16±0.98***</td>
<td>-0.93±0.41†</td>
<td>-0.78±0.52</td>
<td>-3.73±2.74</td>
</tr>
<tr>
<td>0.5 (n=22)</td>
<td>14.53±1.61***</td>
<td>-6.29±1.39***</td>
<td>-5.80±1.30***</td>
<td>-1.26±0.52†</td>
<td>-1.14±0.60***</td>
<td>-2.77±4.33</td>
</tr>
<tr>
<td>1 (n=32)</td>
<td>17.28±2.31***</td>
<td>-7.32±1.04***</td>
<td>-6.59±1.05***</td>
<td>-1.63±0.50†</td>
<td>-2.15±0.55***</td>
<td>-1.64±3.43</td>
</tr>
<tr>
<td>10 (n=30)</td>
<td>33.01±3.63***</td>
<td>-9.15±1.28***</td>
<td>-8.73±1.20***</td>
<td>-2.68±0.67***</td>
<td>-3.86±0.89***</td>
<td>-5.86±5.25</td>
</tr>
<tr>
<td>100 (n=19)</td>
<td>59.51±5.23***</td>
<td>-18.37±1.80***</td>
<td>-19.01±1.93***</td>
<td>-5.50±0.83***</td>
<td>-1.44±0.65†</td>
<td>-6.36±6.93</td>
</tr>
<tr>
<td>1000 (n=24)</td>
<td>40.29±3.96***</td>
<td>-14.50±1.96***</td>
<td>-14.84±2.02***</td>
<td>-4.29±0.57***</td>
<td>-2.95±0.44***</td>
<td>-2.99±4.21</td>
</tr>
<tr>
<td>10000 (n=29)</td>
<td>49.74±3.67***</td>
<td>-15.14±1.74***</td>
<td>-15.44±2.03***</td>
<td>-4.20±0.66***</td>
<td>-4.56±0.82***</td>
<td>2.42±4.48</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. * P < 0.05, ** P < 0.005 and *** P < 0.0001 (paired t-test, pre adrenaline vs. post adrenaline). The ‘–’ sign means that the value had decreased from its corresponding pre-drug administration. n represents the number of cells used in each experiment.
Table S6. Percentage change in AP characteristic in HCMM-CMs at different concentration of adrenaline.

<table>
<thead>
<tr>
<th>Adrenaline (nM)</th>
<th>BPM</th>
<th>APD90</th>
<th>APD50</th>
<th>APA</th>
<th>MDP</th>
<th>dVdT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (n=14)</td>
<td>8.09±2.16**</td>
<td>-7.48±1.68**</td>
<td>-6.48±1.24***</td>
<td>-0.27±0.35</td>
<td>0.10±0.46</td>
<td>3.68±3.96</td>
</tr>
<tr>
<td>0.5 (n=15)</td>
<td>15.46±3.48***</td>
<td>-7.87±1.57***</td>
<td>-7.62±1.46***</td>
<td>-1.08±0.52</td>
<td>-0.70±0.63</td>
<td>-3.75±3.60</td>
</tr>
<tr>
<td>1 (n=15)</td>
<td>24.85±3.78***</td>
<td>-11.43±1.65***</td>
<td>-11.52±1.6***</td>
<td>-0.15±0.43</td>
<td>-0.88±0.78</td>
<td>-2.27±6.24</td>
</tr>
<tr>
<td>10 (n=18)</td>
<td>34.37±4.81***</td>
<td>-21.04±1.80***</td>
<td>-21.72±2.06***</td>
<td>-4.45±0.92***</td>
<td>-2.42±1.07*</td>
<td>-5.26±4.80</td>
</tr>
<tr>
<td>100 (n=10)</td>
<td>34.45±5.67***</td>
<td>-11.30±3.21*</td>
<td>-11.98±3.62*</td>
<td>-4.34±0.91***</td>
<td>-1.73±0.46**</td>
<td>3.60±3.92</td>
</tr>
<tr>
<td>1000 (n=10)</td>
<td>37.44±9.95**</td>
<td>-11.96±3.67*</td>
<td>-12.53±3.90*</td>
<td>-3.45±0.91***</td>
<td>-1.22±1.08</td>
<td>-1.08±9.33</td>
</tr>
<tr>
<td>10000 (n=14)</td>
<td>46.18±8.8***</td>
<td>-17.28±2.34***</td>
<td>-17.94±2.49***</td>
<td>-4.17±0.77***</td>
<td>-2.07±0.79*</td>
<td>-13.58±5.60</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. * P < 0.05, ** P < 0.005 and *** P < 0.0001 (paired t-test, pre adrenaline vs. post adrenaline). The ‘–’ sign means that the value had decreased from its corresponding pre drug administration. n represents the number of cells used in each experiment.
Table S7. Correlation between BPM and APD90 and their corresponding percentage changes at different concentrations of adrenaline.

<table>
<thead>
<tr>
<th>nM</th>
<th>n</th>
<th>Δ% BPM</th>
<th>Δ% APD90</th>
<th>n</th>
<th>Δ% BPM</th>
<th>Δ% APD90</th>
<th>n</th>
<th>Δ% BPM</th>
<th>Δ% APD90</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>13</td>
<td>4.99±1.38</td>
<td>10.0±1.37</td>
<td>29</td>
<td>7.73±1.10</td>
<td>6.28±1.11</td>
<td>14</td>
<td>8.09±2.16</td>
<td>7.48±1.68</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>10.09±1.89</td>
<td>7.97±0.78</td>
<td>22</td>
<td>14.53±1.61</td>
<td>6.29±1.39</td>
<td>15</td>
<td>15.46±3.48</td>
<td>7.87±1.57</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>11.71±1.38</td>
<td>11.27±1.32</td>
<td>32</td>
<td>17.28±2.31</td>
<td>7.32±1.04</td>
<td>** r = 0.5184</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>24.85±3.78</td>
<td>11.43±1.65</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>40.66±4.2</td>
<td>17.57±2.53</td>
<td>30</td>
<td>33.01±3.63</td>
<td>9.15±1.28</td>
<td># r = -0.5037</td>
<td># r = -0.4371</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>18</td>
<td>34.37±4.81</td>
<td>21.04±1.80</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>47.67±4.94</td>
<td>14.53±1.99</td>
<td>19</td>
<td>59.51±5.23</td>
<td>18.37±1.80</td>
<td>10</td>
<td>34.45±5.67</td>
<td>11.30±3.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td># r = -0.7259</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td># r = -0.6630</td>
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<tr>
<td>1000</td>
<td>11</td>
<td>59.60±9.42</td>
<td>17.38±1.94</td>
<td>24</td>
<td>40.29±3.96</td>
<td>14.50±1.96</td>
<td>10</td>
<td>37.44±9.95</td>
<td>11.96±3.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td># r = -0.6157</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td># r = -0.6084</td>
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<tr>
<td>10000</td>
<td>14</td>
<td>68.67±8.29</td>
<td>22.44±2.64</td>
<td>29</td>
<td>49.74±3.67</td>
<td>15.14±1.74</td>
<td>14</td>
<td>46.18±8.8</td>
<td>17.28±2.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td># r = -0.6304</td>
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<td></td>
<td></td>
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</tr>
</tbody>
</table>

Data are presented as mean±SEM. * P < 0.05, ** P < 0.005 (Pearson’s correlation test between Δ% BPM vs. Δ% APD90) and * P < 0.05, ** P < 0.005 (Pearson’s correlation test between BPM vs. Δ% BPM or APD90 vs. Δ% APD90). ‘r’ represents the correlation coefficient where ‘–’ sign means inverse relationship and vice versa. n represent the number of cells used in each experiment.
Table S8. DAD rate (1/min) in hiPSC-CMs. Frequency of DADs was calculated as total number of DADs/total number of APs.

<table>
<thead>
<tr>
<th>nM</th>
<th>n</th>
<th>Baseline</th>
<th>Adrenaline</th>
<th>Washout</th>
<th>n</th>
<th>Baseline</th>
<th>Adrenaline</th>
<th>Washout</th>
<th>n</th>
<th>Baseline</th>
<th>Adrenaline</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>13</td>
<td>0.29±0.20</td>
<td>0.89±0.34</td>
<td>1.0±0.74</td>
<td>29</td>
<td>7.16±2.26**</td>
<td>6.85±2.18</td>
<td>6.24±2.27</td>
<td>14</td>
<td>4.59±1.65</td>
<td>3.72±1.38</td>
<td>2.08±1.08</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0.5±0.15</td>
<td>0.73±0.66</td>
<td>1.26±0.73</td>
<td>24</td>
<td>6.41±2.78</td>
<td>7.93±3.1</td>
<td>5.74±2.72</td>
<td>15</td>
<td>3.9±1.98</td>
<td>4.69±2.18</td>
<td>3.09±1.93</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>1.5±0.8</td>
<td>2.0±1.13</td>
<td>2.4±1.0</td>
<td>32</td>
<td>6.19±2.49</td>
<td>5.3±1.81</td>
<td>5.09±1.73</td>
<td>15</td>
<td>6.6±2.10</td>
<td>4.35±1.61</td>
<td>4.05±2.05</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>31</td>
<td>6.36±2.16*</td>
<td>5.72±1.86</td>
<td>4.0±1.72</td>
<td>18</td>
<td>1.78±0.53#</td>
<td>3.97±2.8</td>
<td>4.03±2.18</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>1.43±1.3</td>
<td>0.60±0.42</td>
<td>0.24±0.17</td>
<td>19</td>
<td>2.26±0.98</td>
<td>0.97±0.9</td>
<td>1.27±0.7</td>
<td>11</td>
<td>2.98±1.17</td>
<td>1.23±0.72</td>
<td>2.08±0.96</td>
</tr>
<tr>
<td>1000</td>
<td>11</td>
<td>0.98±0.7</td>
<td>0.37±0.35</td>
<td>0.55±0.48</td>
<td>24</td>
<td>2.75±1.05</td>
<td>1.65±0.7</td>
<td>2.48±1.21</td>
<td>10</td>
<td>5.65±2.77</td>
<td>1.18±0.60</td>
<td>0.94±0.52</td>
</tr>
<tr>
<td>10000</td>
<td>14</td>
<td>1.09±0.6</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>29</td>
<td>3.53±1.19</td>
<td>1.56±0.57**</td>
<td>2.07±0.63**</td>
<td>14</td>
<td>3.33±1.19</td>
<td>1.43±0.65</td>
<td>1.34±0.75</td>
</tr>
</tbody>
</table>

DAD rates did not change significantly during and immediately after administration of different concentrations of adrenaline from baseline in WT-CMs, HCMT-CMs and HCMM-CMs. (ns: Friedman, posthoc Dunn test). * or # P<0.05, **P<0.005 (WT-CMs Vs HCMT-CMs or HCMM-CMs; Kruskal Wallis, post hoc Dunn test). Data are presented as mean±SEM. n represents the number of cells used in each experiment.
Table S9. Phase 3 EAD rate (1/10min) in hiPSC-CMs. Frequency of phase 3 EAD rate was calculated as total number of Phase 3 EAD/total time.

<table>
<thead>
<tr>
<th></th>
<th>WT-CMs</th>
<th></th>
<th></th>
<th></th>
<th>HCMT-CMs</th>
<th></th>
<th></th>
<th></th>
<th>HCMM-CMs</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
<td>Washout</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
<td>Washout</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>-----</td>
<td>-----------------</td>
<td>-------</td>
<td>----------</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>13</td>
<td>0.0±0.0</td>
<td>0.7±0.4</td>
<td>0.8±0.8</td>
<td>29</td>
<td>0.1±0.1</td>
<td>3.8±2.5</td>
<td>2.1±1.2</td>
<td>14</td>
<td>0.3±0.3</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>10</td>
<td>0.0±0.0</td>
<td>0.3±0.3</td>
<td>0.0±0.0</td>
<td>24</td>
<td>3.8±1.7</td>
<td>2.3±1.4</td>
<td>1.4±0.7</td>
<td>15</td>
<td>0.6±0.6</td>
<td>1.5±0.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>17</td>
<td>0.2±0.2</td>
<td>1.0±0.6</td>
<td>0.0±0.0</td>
<td>32</td>
<td>1.6±0.5</td>
<td>10.0±4.8</td>
<td>7.7±3.6*</td>
<td>15</td>
<td>1.0±1.0</td>
<td>0.7±0.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.5±0.5</td>
<td>31</td>
<td>12.9±8.7</td>
<td>14.6±11.7</td>
<td>7.2±6.5</td>
<td>18</td>
<td>0.3±0.3</td>
<td>0.5±0.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>12</td>
<td>0.3±0.3</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>19</td>
<td>2.0±1.2</td>
<td>0.7±0.5</td>
<td>1.0±0.6</td>
<td>11</td>
<td>0.9±0.9</td>
<td>0.7±0.5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>11</td>
<td>0.4±0.4</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>24</td>
<td>0.8±0.6</td>
<td>0.9±0.5</td>
<td>1.0±0.5</td>
<td>10</td>
<td>2.6±1.1</td>
<td>1.2±0.8</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>14</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>29</td>
<td>0.5±0.3*</td>
<td>1.1±0.5</td>
<td>1.1±0.7</td>
<td>14</td>
<td>3.9±1.5*</td>
<td>0.5±0.3</td>
</tr>
</tbody>
</table>

Phase 3 EAD rate did not change significantly during and immediately after administration of different concentrations of adrenaline from baseline in WT-CMs, HCMT-CMs and HCMM-CMs. (ns: Friedman, post hoc Dunn test). * or # P<0.05 (WT-CMs Vs HCMT-CMs or HCMM-CMs; Kruskal Wallis, post hoc Dunn test). Data are presented as mean±SEM. n represents the number of cells used in each experiment.
**Table S10.** Categorical analysis of occurrence of burst in hiPSC-CMs. Frequency of burst was calculated as total number of burst/total number of cells.

<table>
<thead>
<tr>
<th></th>
<th>WT-CMs</th>
<th></th>
<th>HCMT-CMs</th>
<th></th>
<th>HCMM-CMs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
<td>Washout</td>
<td>n</td>
<td>Baseline</td>
</tr>
<tr>
<td>nM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>13</td>
<td>23% (3)</td>
<td>8% (1)</td>
<td>0% (0)</td>
<td>29</td>
<td>0% (0)</td>
</tr>
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<td>24</td>
<td>13% (3)</td>
</tr>
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<td>12% (2)</td>
<td>18% (3)</td>
<td>12% (2)</td>
<td>32</td>
<td>0% (0)</td>
</tr>
<tr>
<td>10</td>
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<td>0% (0)</td>
<td>13% (1)</td>
<td>31</td>
<td>26% (8)</td>
</tr>
<tr>
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<td>0% (0)</td>
<td>0% (0)</td>
<td>19</td>
<td>5% (1)</td>
</tr>
<tr>
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<td>11</td>
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<td>0% (0)</td>
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<td>24</td>
<td>0% (0)</td>
</tr>
<tr>
<td>10000</td>
<td>14</td>
<td>7% (1)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>29</td>
<td>7% (2)</td>
</tr>
</tbody>
</table>

Data are presented as percentages. The percentages were rounded off to the whole number. n represents the number of cells used in each experiment. The value in parenthesis represents the number of burst occurred in each experiment.
Table S11. Occurrence of QES-EAD in hiPSC-CMs. Frequency of QES-EAD was calculated as total number of QES-EAD/total number of cells.

<table>
<thead>
<tr>
<th>WT-CMs</th>
<th></th>
<th></th>
<th></th>
<th>HCMT-CMs</th>
<th></th>
<th></th>
<th></th>
<th>HCMM-CMs</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>nM</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
<td>Washout</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
<td>Washout</td>
<td>n</td>
<td>Baseline</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>0.1</td>
<td>13</td>
<td>0% (0)</td>
<td>8% (1)</td>
<td>8% (1)</td>
<td>29</td>
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<td>14% (4)</td>
<td>0% (0)</td>
<td>14</td>
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</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0% (0)</td>
<td>0% (0)</td>
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<td>24</td>
<td>0% (0)</td>
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<td>15</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
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<td>0% (0)</td>
<td>0% (0)</td>
<td>12% (2)</td>
<td>32</td>
<td>6% (2)</td>
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<td>3% (1)</td>
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</tr>
<tr>
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<td>8</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>13% (1)</td>
<td>31</td>
<td>0% (0)</td>
<td>6% (2)</td>
<td>0% (0)</td>
<td>18</td>
<td>6% (1)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>100</td>
<td>12</td>
<td>8% (1)</td>
<td>8% (1)</td>
<td>8% (1)</td>
<td>19</td>
<td>0% (0)</td>
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<td>0% (0)</td>
<td>11</td>
<td>0% (0)</td>
<td>9% (1)</td>
</tr>
<tr>
<td>1000</td>
<td>11</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>24</td>
<td>0% (0)</td>
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<td>4% (1)</td>
<td>10</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
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<td>14</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>29</td>
<td>0% (0)</td>
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<td>3% (1)</td>
<td>14</td>
<td>7% (1)</td>
<td>0% (0)</td>
</tr>
</tbody>
</table>

Data are presented as percentage. The percentages were rounded off to the whole number. n represents the number of cells used in each experiment. The value in the parenthesis represents the number of QES-EAD arrhythmia occurred.
### Table S12. Occurrence of VTs in HCMT-CMs.

Three types of VTs were observed in HCMT-CMs.

<table>
<thead>
<tr>
<th>nM</th>
<th>Baseline</th>
<th>Adrenaline</th>
<th>Washout</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1  (n=29)</td>
<td>0</td>
<td>1/29 1:NRVT</td>
<td>0</td>
</tr>
<tr>
<td>0.5  (n=24)</td>
<td>1/24 1: NSVT</td>
<td>3/24 1: NSVT 2: SVT</td>
<td>3/24 2: NSVT 1: SVT</td>
</tr>
<tr>
<td>1    (n=32)</td>
<td>0</td>
<td>3/32 3: NSVT</td>
<td>2/32 2: NSVT</td>
</tr>
<tr>
<td>10   (n=31)</td>
<td>0</td>
<td>1/31 1:SVT 2: NSVT</td>
<td>3/31 2: NSVT 1: NRVT</td>
</tr>
<tr>
<td>100  (n=19)</td>
<td>0</td>
<td>0</td>
<td>1/19 1: NSVT</td>
</tr>
<tr>
<td>1000 (n=24)</td>
<td>0</td>
<td>0</td>
<td>1/24 1: NSVT</td>
</tr>
<tr>
<td>10000 (n=29)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

n represents the number of cells used in each experiment.

SVT: Sustained VT

NSVT: Non-sustained VT

NRVT: Non-recovered VT
Table S13. Effect of various concentrations of adrenaline in variabilities in WT-CMs.

<table>
<thead>
<tr>
<th>Adrenaline</th>
<th>Δ%SD1</th>
<th>Δ%SD2</th>
<th>Δ%SDRR</th>
<th>Δ%SDSD</th>
<th>Δ%STV-APD90</th>
<th>Δ%STV-APD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 nM (n=11)</td>
<td>10.84±12.52</td>
<td>-3.16±7.40</td>
<td>1.17±8.26</td>
<td>10.84±12.52</td>
<td>-6.50±8.74</td>
<td>3.70±7.40</td>
</tr>
<tr>
<td>0.5 nM (n=8)</td>
<td>-1.83±10.08</td>
<td>-12.22±7.29 ( ^* )</td>
<td>-9.69±6.80</td>
<td>-1.83±10.08</td>
<td>-9.00±6.42</td>
<td>-4.00±5.14</td>
</tr>
<tr>
<td>1 nM (n=14)</td>
<td>-2.11±11.77</td>
<td>2.64±11.90</td>
<td>-0.81±9.44</td>
<td>-2.11±11.77</td>
<td>-7.53±4.47</td>
<td>-7.52±5.14</td>
</tr>
<tr>
<td>10 nM (n=13)</td>
<td>-27.39±5.34 ( ^{**} )</td>
<td>-28.84±7.95 ( ^{**} )</td>
<td>-30.88±6.52 ( ^{**} )</td>
<td>-27.39±5.34 ( ^{**} )</td>
<td>-23.81±6.36 ( ^{**} )</td>
<td>-23.28±5.87 ( ^{**} )</td>
</tr>
<tr>
<td>100 nM (n=11)</td>
<td>-36.09±7.90</td>
<td>-35.21±8.19 ( ^{*} )</td>
<td>-35.76±7.70 ( ^{*} )</td>
<td>-36.09±7.90</td>
<td>-23.15±6.27 ( ^{**} )</td>
<td>-23.77±6.12 ( ^{**} )</td>
</tr>
<tr>
<td>1uM (n=7)</td>
<td>-42.46±8.81 ( ^{*} )</td>
<td>-41.45±12.48</td>
<td>-44.36±11.27</td>
<td>-42.46±8.81 ( ^{*} )</td>
<td>-25.85±5.74 ( ^{**} )</td>
<td>-25.16±6.11 ( ^{**} )</td>
</tr>
<tr>
<td>10 uM (n=10)</td>
<td>-49.66±8.02 ( ^{***} )</td>
<td>-52.45±9.70 ( ^{*} )</td>
<td>-55.91±8.02 ( ^{**} )</td>
<td>-55.65±5.96 ( ^{**} )</td>
<td>-35.99±8.20 ( ^{**} )</td>
<td>-36.36±8.47 ( ^{**} )</td>
</tr>
</tbody>
</table>

The percentage change was calculated with respect to baseline (pre-adrenaline) value. Data are presented as mean±SEM. * \( P < 0.05 \), ** \( P < 0.005 \) and *** \( P < 0.0001 \) (paired t-test, pre adrenaline vs. post adrenaline) n represent the number of cells used in each experiment. The ‘–’ sign means that the value had decreased from its corresponding pre-drug administration.
Table S14. Effect of various concentrations of adrenaline in variabilities in HCMT-CMs.

<table>
<thead>
<tr>
<th>Adrenaline</th>
<th>∆%SD1</th>
<th>∆%SD2</th>
<th>∆%SDRR</th>
<th>∆%SDSD</th>
<th>∆%STV-APD90</th>
<th>∆%STV-APD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 nM (n=17)</td>
<td>22.14±9.90</td>
<td>23.96±12.36</td>
<td>22.93±11.21</td>
<td>22.14±9.90</td>
<td>2.59±6.98</td>
<td>9.32±5.24</td>
</tr>
<tr>
<td>1 nM (n=16)</td>
<td>-16.48±4.79*</td>
<td>-18.58±6.92*</td>
<td>-18.47±5.75**</td>
<td>-16.48±4.79**</td>
<td>-3.44±3.92</td>
<td>-4.04±4.05</td>
</tr>
<tr>
<td>10 nM (n=22)</td>
<td>-14.43±9.58</td>
<td>-17.09±12.22</td>
<td>-16.64±11.32*</td>
<td>-14.43±9.58*</td>
<td>-5.64±3.99*</td>
<td>-8.27±3.59*</td>
</tr>
<tr>
<td>100 nM (n=13)</td>
<td>-33.30±4.93*</td>
<td>-2.75±12.97</td>
<td>-14.68±8.84</td>
<td>-33.30±4.93*</td>
<td>-27.50±5.36</td>
<td>-26.07±5.18</td>
</tr>
<tr>
<td>1uM (n=20)</td>
<td>-33.77±5.09***</td>
<td>-41.07±6.08***</td>
<td>-39.46±5.18***</td>
<td>-33.77±5.09***</td>
<td>-23.89±4.44**</td>
<td>-24.22±4.22**</td>
</tr>
<tr>
<td>10 uM (n=21)</td>
<td>-37.08±6.57***</td>
<td>-36.69±6.31***</td>
<td>-37.50±6.26***</td>
<td>-37.08±6.57***</td>
<td>-19.58±5.22*</td>
<td>-22.18±5.68*</td>
</tr>
</tbody>
</table>

The percentage change was calculated with respect to baseline (pre-adrenaline) value. Data are represented as mean±SEM. * P < 0.05, ** P < 0.005 and *** P < 0.0001 (paired t-test, pre adrenaline vs. post adrenaline) n represents the number of cells used in each experiment. The ‘–’ sign means that the value had decreased from its corresponding pre-drug administration.
Table S15. Effect of various concentration of adrenaline in variabilities in HCMM-CMs.

<table>
<thead>
<tr>
<th>Adrenaline</th>
<th>Δ%SD1</th>
<th>Δ%SD2</th>
<th>Δ%SDRR</th>
<th>Δ%SDSD</th>
<th>Δ%STV-APD90</th>
<th>Δ%STV-APD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 nM (n=11)</td>
<td>30.70±15.23</td>
<td>7.27±11.57</td>
<td>12.85±11.54</td>
<td>30.70±15.23</td>
<td>-1.74±5.51</td>
<td>0.21±5.72</td>
</tr>
<tr>
<td>0.5 nM (n=13)</td>
<td>-6.46±11.21</td>
<td>0.92±10.58</td>
<td>-1.63±10.32</td>
<td>-6.46±11.21</td>
<td>-10.38±4.61</td>
<td>-10.08±5.17</td>
</tr>
<tr>
<td>1 nM (n=8)</td>
<td>-10.05±14.04</td>
<td>-17.34±18.51</td>
<td>-16.45±17.03</td>
<td>-10.05±14.04</td>
<td>-23.33±6.20</td>
<td>-23.03±5.80</td>
</tr>
<tr>
<td>100 nM (n=8)</td>
<td>-35.90±7.13*</td>
<td>-25.22±8.13</td>
<td>-29.75±7.18*</td>
<td>-35.90±7.13*</td>
<td>-21.86±6.63*</td>
<td>-23.17±6.55*</td>
</tr>
<tr>
<td>1uM (n=7)</td>
<td>-42.16±11.75</td>
<td>-47.83±9.49*</td>
<td>-47.75±9.09*</td>
<td>-42.16±11.75</td>
<td>-20.92±8.87</td>
<td>-20.75±8.65</td>
</tr>
<tr>
<td>10 uM (n=9)</td>
<td>-33.78±5.81*</td>
<td>-40.39±5.41*</td>
<td>-37.66±5.38*</td>
<td>-33.78±5.81*</td>
<td>-11.58±8.15</td>
<td>-12.42±7.92</td>
</tr>
</tbody>
</table>

The percentage change was calculated with respect to baseline (pre-adrenaline) value. Data are presented as mean±SEM. * P < 0.05, (paired t-test, pre adrenaline vs. post adrenaline) n represents the number of cells used in each experiment. The ‘–’ sign means that the value had decreased from its corresponding pre drug administration.
Figure S9. Experimental protocol for adrenaline and bisoprolol testing. After the stable recording, adrenaline alone (ESa) was passed for 1-2 minute after which extracellular solution containing both adrenaline and bisoprolol (ESab) was passed. Subsequently, extracellular solution only with bisoprolol (ESb) was used. To study the efficacy of bisoprolol, APs were chosen from recording after minimum of 3-minute perfusion of solution with adrenaline and bisoprolol (ESab). Furthermore, to study the potency of bisoprolol in washing out condition of adrenaline, maximum up to 300ms immediately after the adrenaline being washout were selected for analysis.
Simultaneous recordings of action potentials and calcium transients from human induced pluripotent stem cell derived cardiomyocytes

Chandra Prajapati1,‡, Risto-Pekka Pölönen1,‡ and Katriina Aalto-Setälä1,2,3,*

ABSTRACT

Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) offer a unique in vitro platform to study cardiac diseases, as they recapitulate many disease phenotypes. The membrane potential (V_m) and intracellular calcium (Ca^{2+}) transient (CaT) are usually investigated separately, because incorporating different techniques to acquire both aspects concurrently is challenging. In this study, we recorded V_m and CaT simultaneously to understand the interrelation between these parameters in hiPSC-CMs. For this, we used a conventional patch clamp technique to record V_m, and synchronized recording of V_m and calcium transient (CaT) broadens the understanding of the interrelation between V_m and CaT and could be used to elucidate the mechanisms underlying arrhythmia in cardiac disease condition.

KEY WORDS: hiPSC-CMs, Membrane potential, Calcium transient, Delayed after depolarization, Early after depolarization

INTRODUCTION

The discovery of human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007) from somatic cells and their ability to differentiate into cardiomyocytes (CMs) (hiPSC-CMs) provides a robust platform to study genetic cardiac diseases (Kujala et al., 2012; Lahti et al., 2012; Kivihal et al., 2015; Penttininen et al., 2015; Ojala et al., 2016; Prajapati et al., 2018; Izhaki et al., 2012; Ma et al., 2015; Spencer et al., 2014) and for drug screening (Li et al., 2012; Spencer et al., 2014). Thus, these hiPSC-CMs closely mimic cardiac functionality and have already recapitulated many genetic cardiac diseases such as LQT1 (Kivihal et al., 2015; Kuusela et al., 2017; Ma et al., 2015), LQT2 (Lahti et al., 2012; Spencer et al., 2014), CPVT (Kujala et al., 2012; Izhaki et al., 2012; Penttininen et al., 2015; Pölönen et al., 2018; Ahol et al., 2017) and HCM (Ojala et al., 2016; Prajapati et al., 2018; Földes et al., 2014; Lan et al., 2013; Han et al., 2014). In CMs, the contraction is driven by action potential (AP) induced release of intracellular Ca^{2+}. Inward current through sodium channels (I_{Na}) starts the rapid depolarization of the CM cell membrane, which triggers inward current through L-type calcium channels (I_{Ca,L}). This leads to Ca^{2+} induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) via ryanodine receptors. Ca^{2+} binds to sarcomeres, the contractile units of myofibers, and allows the movement of myofilaments and contractile motion. The cell membrane is then repolarized by several repolarizing potassium currents and Ca^{2+} is reloaded into SR via SR Ca^{2+} ATPase. (Kane et al., 2015)

The presence of ion channels plays a major role in shaping AP and Ca^{2+} dynamics (Bartos et al., 2015). The membrane potential (V_m) and intracellular Ca^{2+} concentration ([Ca^{2+}]_i) are the most crucial elements in the normal physiology and also in arrhythmias in cardiology. Ca^{2+} ion plays a major role in activation and modulation of contraction in CMs. The measurement of [Ca^{2+}]_i, using a Ca^{2+} indicator during contraction and relaxation of CMs reflects the SR Ca^{2+} release and uptake. Furthermore, [Ca^{2+}]_i influences the shape and duration of AP via Ca^{2+}-sensitive ionic channels such as I_{Na}, Na^{+}-Ca^{2+} exchanger (NCX) and Ca^{2+}-activated nonselective cation channels, thereby modifying the electrophysiological properties, for example, the refractoriness and membrane depolarization rate (Wu et al., 2005; Bers, 2002). Conversely, V_m can influence the Ca^{2+} spark and waves (Sato et al., 2014). Thus, the V_m and Ca^{2+} cycle are highly interdependent and bidirectionally coupled (Omichi et al., 2004). Only few studies have been focused on simultaneous recording of V_m and calcium transient (CaT) from the same hiPSC-CMs (Lee et al., 2012; Spencer et al., 2014). Thus, this hinders the understanding of the interaction between V_m and CaT from the same hiPSC-CMs. To improve the understanding of the complex dynamics and mechanisms underlying arrhythmias, it is ideal to analyze V_m and CaT simultaneously from the same cell. In this study, we performed simultaneous V_m and CaT recordings from the same hiPSC-CMs to investigate in more detail the correlation between these parameters.

RESULTS

Immunocytochemistry

Immunostaining experiments were performed to confirm the presence of different cardiac proteins in hiPSC-CMs. Dissociated hiPSC-CMs
were imaged with a confocal microscope with z-stack. Immunostainings of hiPSC-CMs showed the positive staining of cardiac troponin T (cTnT) (green), CaV1.2 (red) and cardiac ryanodine receptor (RyR2) (red) (Fig. 1). These immunostaining images show the homogenous distribution of ICa,L and RyR2 in hiPSC-CMs.

**Voltage-gated ionic current hiPSC-CMs**

The presence of various voltage-gated ionic currents in hiPSC-CMs were confirmed using voltage clamp techniques. Fig. 2 summarizes the representative AP and different ionic currents recorded from hiPSC-CMs. The fast INa is responsible for the rapid upstroke.
phase in AP, and peak \( I_{\text{Na}} \) current density was \(-23.1\pm5.2\) pA/pF \((n=15; \text{Fig. S1A})\). Following rapid depolarization, transient outward potassium current \((I_{\text{to}})\) starts the repolarization of AP. We found that the peak \( I_{\text{to}} \) current density at 70 mV was \(7.3\pm1.4\) pA/pF \((n=5; \text{Fig. S1B})\). The calcium current \((I_{\text{Ca}})\) is responsible for the plateau phase of AP, and peak \( I_{\text{Ca}} \) current density at 10 mV was \(2.6\pm0.7\) pA/pF \((n=5; \text{Fig. S1C})\). As the plateau phase moves toward more negative membrane potential, mainly two types of potassium currents, rapid rectifier potassium current \((I_{\text{Kr}})\) and slow rectifier potassium current \((I_{\text{Ks}})\), are activated. \( I_{\text{Kr}} \) peak current was calculated at the end of 3 s depolarization test potential and \(I_{\text{Kr}}\) tail current is calculated as peak current in response to step depolarization. The peak and tail current densities of \( I_{\text{Kr}} \) at 0 mV were \(1.0\pm0.3\) and \(1.1\pm0.2\) pA/pF, respectively \((n=5; \text{Fig. S1D})\). Similarly, the peak and tail current densities of \( I_{\text{Ks}} \) at 40 mV were \(0.6\pm0.1\) and \(0.5\pm0.1\) pA/pF, respectively \((n=6; \text{Fig. S1E})\). In addition, inward rectifying potassium current \((I_{\text{K1}})\) is activated during and following the repolarization phase to ensure the terminal repolarization and stable resting membrane potential. The peak \( I_{\text{K1}} \) current density and funny current \((I_{f})\) density at 130 mV were \(-2.5\pm0.6\) pA/pF and \(-3.7\pm1.0\) pA/pF, respectively \((n=6; \text{Fig. S1F-G})\).

**Correlation of CaTs with AP**

Spontaneously beating hiPSC-CMs with visible Ca\(^{2+}\) dynamics under a microscope were chosen for simultaneous recording of APs and CaTs. APs were continuously recorded in gap-free mode, while CaTs were recorded in between for a minimum of 30 s. The majority of the patched cells (~90%) were characterized as ventricular-like hiPSC-CMs. Only a minority of the patched cells were atrial-like (~5%) or nodal-like (~5%) hiPSC-CMs. Examples of APs and CaTs recorded from the same cells representing all three types of hiPSC-CMs are shown in Fig. 3. As shown in Fig. 3, the upstroke of AP was followed by a rapid increase in \([\text{Ca}^{2+}]\). Notably, \([\text{Ca}^{2+}]\) decreased to the minimum diastolic level after the repolarization phase of AP in hiPSC-CMs subtypes. Thus, the CaT at 90% decay (CaT90) was always longer than AP duration (APD90) irrespective of hiPSC-CMs subtypes. Our results showed that the average APD90 and CaT90 in ventricular-like hiPSC-CMs were \(326.3\pm4.5\) ms \((N=37, n=583)\) and \(790.7\pm13.7\) ms \((N=37, n=583)\), respectively. This implies that CaT90s were approximately 2.4 times longer than APD90s (Fig. 3A,D). The average APD90 and CaT90 in atrial-like hiPSC-CMs were \(235.5\pm14\) ms \((N=2, n=35)\) and \(552.8\pm18.3\) ms \((N=2, n=35)\), respectively, and thus CaT90s were approximately 2.3 times longer than APD90s (Fig. 3B,E). The same was observed with nodal-like hiPSC-CMs, the average APD90 and CaT90 were \(195.4\pm3.0\) ms \((N=2, n=49)\) and \(512.5\pm12.4\) ms \((N=2, n=49)\), respectively, therefore CaT90s were 2.6 times longer than APD90s (Fig. 3C,F). We performed the correlation tests between APD90 and CaT90; APD at 50% repolarization (APD50) and CaT at 50% decay (CaT50) and APD50 and time-to-peak of CaT in...
ventricular-like hiPSC-CMs (Fig. 4). Our results demonstrated a positive correlation (N=37, n=583, P<0.0001, Pearson’s correlation test) between these parameters, indicating that CaT parameters and AP parameters are interdependent. The END-2 differentiation technique produces a lower number of atrial-like and nodal-like hiPSC-CMs, thus only small number of those cell types were recorded in this study. Therefore, we did not perform correlation tests in atrial- and nodal-like hiPSC-CMs.

AP and CaT during arrhythmias

During simultaneous recording under baseline conditions, delayed after depolarizations (DADs) were occasionally observed (Fig. 5). DADs were defined as abnormal membrane depolarizations with amplitudes of ≥3% of the preceding AP that occurred after completion of the repolarization. Similarly, early after depolarizations (EADs) were defined as abnormal firings either during phase 2 or phase 3 of AP. At baseline, each DAD and phase 3 EAD recorded in AP were compared to their corresponding CaT of the same cell. Interestingly, 33 DADs in the Vm recording corresponded to [Ca2+]i elevation (Fig. 5A,D). However, 22 DADs presented changes in Vm without any corresponding change in [Ca2+]i, i.e. DAD was observed in AP recording, but no change was observed in [Ca2+]i (Fig. 5B,E; Fig. S2A). Furthermore, we performed a correlation test (Fig. S2A) on those 33 DADs with similar observations in Vm and CaT between relative DAD amplitude (i.e. % of DAD amplitude with respect to APA) and the corresponding relative CaT amplitude (i.e. % amplitude of [Ca2+]i elevation with respect to CaT amplitude). The results demonstrated a positive correlation between these two parameters (P=0.01, Pearson’s correlation test), implying that the amplitude of DADs was dependent on the amplitude of elevated [Ca2+]i. Also, we compared the amplitude of DADs with and without corresponding elevation in [Ca2+]i (Fig. S2B). We found that the average DAD amplitude (10.4±0.6 mV; n=33) with corresponding elevation in [Ca2+]i was significantly higher than the average DAD amplitude (6.5±0.5 mV; n=26) without corresponding [Ca2+]i elevation (P<0.0001, student t-test). In addition, phase 3 EADs were recorded during the simultaneous recording of Vm and CaT during baseline conditions (n=2, Fig. 5C,F) and elevated [Ca2+]i, was observed in CaT at the same time as in phase 3 EADs observed in Vm.

Effect of ion channel blockers on AP and CaT

Initially, simultaneous APs and CaTs were recorded in a normal extracellular solution. To test whether ICa,L is important for the spontaneous beating of hiPSC-CMs, 5 µM nimodipine was used (Fig. 6A). This caused the cessation of both AP and CaT in all of the cells tested (N=3, Fig. 6B). E-4031 (650 nM) was used to block the ICa_L in hiPSC-CMs, and after 1 min of exposure to E4031, four types of responses were observed (Fig. 7). Firstly, 10% (N=1/10) of cells showed prolongation of both AP and CaT, but there was no occurrence of EAD (Fig. 7A,D). The maximum diastolic potential (MDP) was decreased to −50 mV and the APD90/50 ratio became 1.4±0.01 (n=12), meaning APs became slightly more triangular. In addition, the APD90 and CaT90 were 420.9±3.6 (n=12) and 891.7±8.7 (n=12) respectively; increasing by 72% and 77%, respectively, from their baseline values. Secondly, 40% (N=4/10) of cells showed prolongation of both AP and CaT duration and this eventually led to phase 2 EADs (Fig. 7B,E). The CaT90s were extended in proportion to the prolongation of APD90s and CaT followed the shape of Vm. In EADs, the average APD90 and CaT90 were 2334.3±508.2 ms and 2471.1±508.7 ms (n=27), respectively. Notably, the CaT90 was approximately 1.1 times longer than the APD90. Thus, the gaps between Vm and [Ca2+]i were closer during the terminal repolarization in phase 2 EAD episodes. Thirdly, 30% (N=3/10) of cells had depolarized MDP and increased in beating frequency, i.e. an oscillation configuration with or without the occurrence of phase 2 and phase 3 EADs (Fig. 7C,F). The MDP was decreased to 42.7±3.4% (N=3) and beating frequency was increased by 110.6±43.1% (N=3). In these cases, the APD90s were increased by 41% and the average value was 355.2±3.7 (n=60). In contrast, the CaT90s were decreased by 2.4% and the average value was 547.3±6.9 (n=60). Although CaT90s were decreased from baseline, APD90s were still shorter than CaT90 in oscillation conditions. Moreover, the APD90/APD50 ratio was increased to 1.8±0.01 (n=60), indicating that AP shape became more triangular than in baseline conditions. Finally, 20% (N=2/10) of cells showed a cessation of beating, with minimal Vm and [Ca2+]i fluctuation (data not shown).

DISCUSSION

In this study, we found that, in hiPSC-CMs: (1) voltage-gated ionic currents are functional; (2) there are strong correlations between AP and CaT parameters; (3) the amplitude of DADs was dependent on the amplitude of elevated [Ca2+]i. Also, we compared the amplitude of DADs with and without corresponding elevation in [Ca2+]i; (4) we performed a correlation test (Fig. S2A) on those 33 DADs with similar observations in Vm and CaT between relative DAD amplitude (i.e. % of DAD amplitude with respect to APA) and the corresponding relative CaT amplitude (i.e. % amplitude of [Ca2+]i elevation with respect to CaT amplitude). The results demonstrated a positive correlation between these two parameters (P=0.01, Pearson’s correlation test), implying that the amplitude of DADs was dependent on the amplitude of elevated [Ca2+]i. Also, we compared the amplitude of DADs with and without corresponding elevation in [Ca2+]i (Fig. S2B). We found that the average DAD amplitude (10.4±0.6 mV; n=33) with corresponding elevation in [Ca2+]i was significantly higher than the average DAD amplitude (6.5±0.5 mV; n=26) without corresponding [Ca2+]i elevation (P<0.0001, student t-test). In addition, phase 3 EADs were recorded during the simultaneous recording of Vm and CaT during baseline conditions (n=2, Fig. 5C,F) and elevated [Ca2+]i, was observed in CaT at the same time as in phase 3 EADs observed in Vm.
parameters and CaT parameters; (3) DADs observed in $V_m$ recordings are mostly characterized by elevation in $[Ca^{2+}]_i$; (4) blocking of $I_{Kr}$ causes variable responses, including prolongation of both APD and CaT duration, occurrence of EAD and even cessation of beating. The AP recordings from hiPSC-CMs exhibit all phases of AP and current densities of different voltage-gated channels measured in the present study are comparable to earlier studies in hiPSC-CMs (Han et al., 2014; Spencer et al., 2014; Lee et al., 2016; Ma et al., 2015). The depolarization of $V_m$ causes the opening of $I_{Ca,L}$, and facilitates the main pathway for Ca$^{2+}$ entry for CICR (Kane et al., 2015). However, in this study, blocking the $I_{Ca,L}$ resulted in spontaneous beating ending, which is consistent with earlier studies (Itzhaki et al., 2011; Spencer et al., 2014), indicating the importance of $I_{Ca,L}$ in the normal function of CMs. The Ca$^{2+}$-activated transient currents influence the characteristics of normal AP (Lafamme and Becker, 1996). Similarly, abnormal intracellular Ca$^{2+}$ handling cause the dysfunction of contraction/relaxation and arrhythmia in diseased CMs (Kujala et al., 2012; Penttinen et al., 2015; Ojala et al., 2016).

This study demonstrated that in normal conditions, the CaT90s were twice as long in duration than APD90s in hiPSC-CMs. In an earlier study of simultaneous voltage and Ca$^{2+}$ mapping using Fura-4F in the hiPSC-CMs monolayer, the CaT90s were also observed to be longer than APD90s (approximately 1.2 times) (Lee et al., 2012). A possible reason for the larger difference in our study compared to earlier studies might be due to the Ca$^{2+}$ indicators: there was low-affinity ratiometric Fura-4F in previous studies as opposed to high-affinity non-ratiometric Fluo-4 as Ca$^{2+}$ dye in our study. Ca$^{2+}$ dye with high-affinity (Fluo-4) had significantly longer CaT duration than Ca$^{2+}$ dye with low-affinity (Fura-4F) (Kong and Fast, 2014; Fast et al., 2004).

Our earlier studies (Ojala et al., 2016; Kujala et al., 2012; Prajapati et al., 2018) and studies by other groups (Itzhaki et al., 2012; Liang et al., 2013; Lan et al., 2013) have shown that control hiPSC-CMs also occasionally exhibited arrhythmias (DADs and phase 3 EADs) under baseline conditions. However, phase 2 EADs were not observed in control hiPSC-CMs at baseline, but were commonly observed in disease conditions such as LQT1 and LQT2 (Kuusela et al., 2017; Spencer et al., 2014; Ma et al., 2015). In addition, we demonstrated that DADs and EADs could take place in the same CMs (Ojala et al., 2016; Prajapati et al., 2018) particularly because spontaneous SR Ca$^{2+}$ release plays a role in the occurrence of both DADs and EADs (Volders et al., 1997). The well-established mechanism of DADs is that the spontaneous release of Ca$^{2+}$ from SR induces transient inward current generated either by the activation of NCX, calcium-activated Cl$^{-}$ current or non-selective cationic current (Ko et al., 2017; Verkerk et al., 2000; Schlottauer and Bers, 2000). A similar DAD mechanism was also observed in this study with hiPSC-CMs where elevation in $[Ca^{2+}]_i$ resulted in the rise in $V_m$. However, we also observed events where $[Ca^{2+}]_i$ did not change, although DADs were observed in $V_m$. We postulate three possible explanations for this discrepancy: (1) the
[Ca^{2+}]_{i}$ amplitude got reduced by spatial averaging since the amplitude of DADs without [Ca^{2+}]_{i} elevation was significantly lower, (2) high-affinity Fluo-4 artificially prolongs the CaT duration, which overshadowed the DAD observed nearer to terminal repolarization of AP, or (3) the involvement of Ca^{2+}-independent currents promoting depolarization of V_{m}, and possible mechanisms is still unknown. Consistent with a previous study (Mitsunori et al., 2009), the amplitude of DAD was dependent on the amplitude of [Ca^{2+}]_{i} elevation in our study. Furthermore, the DAD amplitude is also dependent on the sensitivity of resting V_{m} to change in [Ca^{2+}]_{i} i.e. diastolic Ca^{2+}-voltage coupling gain (Mitsunori et al., 2009). On the other hand, APDs and CaTs were prolonged concurrently with the pharmacological blockage of IKr by E-4031; phase 2 EADs were also occasionally observed. During phase 2 EAD, the notable observation was that the CaT always followed the V_{m} during the EAD episodes i.e. CaT was changing corresponding to small V_{m} oscillations. This demonstrates a strong dependency between V_{m} and CaT during this kind of arrhythmic condition. Furthermore, the upstroke of an EAD is generally carried by ICa, and take-off potential of an EAD depends on the complex interplay between the kinetics of ICa and IKs (Chang et al., 2011). The mechanism of phase 2 EAD is that the window current of ICaL overlapping the V_{m} promotes the reactivation of ICa,L and causes EAD to occur (January and Riddle 1989). In addition, an earlier study explained that spontaneous SR Ca^{2+} release during the plateau phase of AP has an essential role in the development of an EAD (Choi et al., 2002). The phase 3 EAD shares the properties of both DADs and phase 2 EADs, but it has its own unique character. Phase 3 is distinguished by the breaking off in the final phase of repolarization of AP. The elevated [Ca^{2+}]_{i} during repolarization enhances the NCX current that could potentially trigger phase 3 EADs (Volders et al., 2000; Maruyama et al., 2012). Our study demonstrates that the CaT was also following the change in V_{m} during the phase 3 EAD, similarly as in the phase 2 EAD. An earlier study using Langendorff rabbit heart reported that the CaT faithfully tracts the V_{m} during faster beating frequency and ventricular tachycardia (Wu et al., 2005). However, there is a possibility that SR Ca^{2+} cycling undergoes an intrinsic dynamic independent of V_{m}. One example of such is during ventricular fibrillation (VF), where CaT is no longer associated with V_{m} (Omichi et al., 2004; Wu et al., 2005). Taken together, CaT and V_{m} are closely associated not only in normal condition, but also in phase 2/3 EADs and in the majority of DADs. However, CaT can dissociate from V_{m}, and go through its own pathway in certain conditions, such as in a VF episode.

**Fig. 6. Effect of 5 µM Nimodipine in spontaneous beating of hiPSC-CMs.**

(A) Baseline recording of action potentials (black, upper trace) and corresponding CaT traces (red, lower trace) from the same hiPSC-CMs. (B) Representative traces of membrane potential (black, upper trace) and CaT (red, lower trace) showing the cessation of spontaneous beating with blockage of calcium channels. (C,D) Action potential amplitudes and CaT amplitudes were normalized to 1.5 and 1 value respectively. Dashed lines represent the 0 mV. (N=3).

**MATERIALS AND METHODS**

**Ethical approval and generation of hiPSC lines**

Approval from the Ethics Committee of Pirkanmaa Hospital District was given to conduct the research on hiPSC lines (Aalto-Setälä R08070). Patients donating skin biopsies gave informed consent in Tays Heart Hospital, Tampere University Hospital, Finland. Two control hiPSC lines, UTA.04602.WT
Fig. 7. Three different responses of 650 nM E-4031 in spontaneous beating of hiPSC-CMs. (A) Representative traces of baseline action potential (black, upper trace) and corresponding CaT (red, lower trace). (B) Prolongation of action potential duration (black, upper trace) and corresponding CaT (red, lower trace). (C) Occurrence of phase 2 EAD (black, upper trace) and corresponding CaT (red, lower trace). (D) Oscillation of membrane potential with EADs (black, upper trace) corresponding with CaT (red, lower trace). (E-H) Action potential amplitudes and CaT amplitudes were normalized to 1.5 and 1 value respectively. Dashed lines represent the 0 mV. Red arrowheads indicate the phase 2 and phase 3 EADs.
(healthy 56-year-old female) and UTA.04511.1WT (healthy 34-year-old male) were used in this study. The UTA.04511.1WT hiPSC line was generated using Sendai vectors, and UTA.04602.0WT was generated by using pMX retroviral vectors without Cre-loxP site. Both hiPSC lines were derived and cultured on mouse embryonic fibroblast (MEF) feeder cell layers (26,000 cells/cm²; CellSystems Biotechnologie Vertrieb GmbH, Troisdorf, Germany) in culture medium containing knockout-dMEM (ko-dMEM) (Gibco) supplemented with 20% knockout serum replacement (ko-SR) (Gibco), 1% nonessential amino acids (NEAA) (Lonza Group Ltd, Basel, Switzerland), 2 mM GlutaMax (Gibco), 50 µM penicillin/streptomycin (Lonza Group Ltd), 0.1 mM 2-mercaptoethanol (Gibco) and 4 ng/ml basic fibroblast growth factor (bFGF) (PeproTech, Rocky Hill, USA). The characterization of these lines were found in previous study (Ojala et al., 2016).

Differentiation into cardiomyocytes and dissociation
Both hiPSC lines were differentiated into cardiomyocytes by co-culturing with Mitomycin C (Sigma-Aldrich) treated mouse visceral endodermal-like cells (END-2) (Hubrecht Institute, Utrecht, Netherlands) (50,000 cells/cm²) as described earlier (Mummery et al., 2003; Ojala et al., 2012). MEF feeder cell layers were removed manually before differentiation. Approximately 30 colonies per well were detached and transferred onto END-2 cells in stem cell culture medium without ko-SR or bFGF and supplemented with 3 mg/ml ascorbic acid (Sigma-Aldrich). Medium was changed after 8, 5 and 12 days of culturing. After 15 days of culturing, 10% ko-SR was included and ascorbic acid was excluded from the culture medium; subsequently, the medium was changed three times per week. Beating areas were cut and washed in Low-Ca buffer [12 ml 1 M NaCl, 0.54 ml 1 M KCl, 0.5 ml 1 M MgSO4, 0.5 ml 1 M Na pyruvate, 2 ml 1 M glucose, 20 ml 0.1 M taurine and 1 ml 1 M HEPES (pH adjusted to 6.9 with NaOH)] and 3 mM 4-AP. From the holding potential of −120 mV for 50 ms and then ramped up to 40 mV with rate of 0.53 V/s. The ICa were measured in the presence of 300 µM CaCl2 to block the Ca2+ current, and HP of −80 mV. Two-step protocol was used: first step was −50 mV for 50 ms to inactivate sodium channels, then to test potential of 60 mV to 70 mV with the step-size of 10 mV was used. The Ito was measured in the presence of 3 mM 4-AP to block Ito, and an HP of −40 mV. To elicit the Ca2+ current, step-protocol from −60 mV to 70 mV with the step-size of 10 mV was used. The Ito was measured in the presence of 3 M NaCl; to block the Ca2+ current, and HP of −80 mV. Two-step protocol was used: first step was −50 mV for 50 ms to inactivate sodium channels, then to test potential of 500 ms duration from −50 mV to 70 mV with step-size of 10 mV. The Ito was measured as 1 µM E4031-sensitive current in the presence of 5 µM nimodipine and 10 µM chromanol 293B-sensitive current in the presence of 5 µM nimodipine and 1 µM E4031 to block Ito, and Ito respectively. Using the HP of −40 mV, step protocol from −20 to 40 mV of 3 s with step size of 20 mV was used. The Ito was measured as 10 µM chromanol 293B-sensitive current in the presence of 5 µM nimodipine and 1 µM E4031 to block Ito, and Ito respectively. Using the HP of −40 mV, step protocol from 0 to 40 mV of 3 s with step-size of 20 mV was used. The Ito and Ito were measured as 2 mM BaCl2 sensitive and insensitive current respectively in the presence of 300 µM CdCl2 and 3 mM 4-AP. From the holding potential of −40 mV, test potential from −140 mV to 0 mV with duration of 700 ms and step-size of 10 mV was used.
Data analysis and statistics
Recorded APs were analyzed with OriginTM 9.1 (OriginLab Corp., Northampton, USA) to extract APD50 and APD90. Voltage clamp data were analyzed using Clampfit software version 10.5 (Molecular Devices LLC). For Ca2+ imaging analysis, a whole cell region of interest (ROI) was drawn. Acquired signal was normalized as ΔF/ΔF0 using LiveAcquisition software (TILL Photonics). The Ca2+ traces were analyzed with Clampfit software to extract Ca2+ transient at 50% and 90% of repolarization (CaT50 and CaT90), and time-to-peak (time taken for CaT to reach its peak intensity). Each AP parameter was compared with its corresponding CaT parameter. Data from both hiPSC lines were combined. The hiPSC-CMs were categorized as ventricular-like and atrial-like CMs when CaT parameter. Data from both hiPSC lines were combined. The hiPSC-CMs both during normal regular beating and in arrhythmic conditions. The simultaneous recording of Vm and CaT allows us to study the intracellular Ca2+ dynamics with respect to changes in Vm in hiPSC-CMs both during normal regular beating and in arrhythmic conditions. The simultaneous recording of Vm and CaT allows us to study the intracellular Ca2+ dynamics with respect to changes in Vm and CaT in more detail. Furthermore, hiPSC-CMs provide a safe and powerful tool to study the cardiac physiology and pathophysiology in vitro.

Study limitation
Although hiPSC-CMs offer a robust platform for in vitro modeling of various genetic cardiac diseases, they have potential limitations because of their intrinsic differences compared to adult CMs. Some of the main ways that they do not fully resemble adult CMs are their rate of t-tubules and low expression of I
t-L. Furthermore, the majority of hiPSC-CMs differen- tiate into ventricular-like cardiomyocytes with our differentiation protocol, thus only limited atrial-like and nodal-like hiPSC-CMs are obtained. This hinders the ability to study the correlation between Vm and CaT in atrial and nodal cardiomyocytes. In addition, technical limitations exist in our measurement. During an AP measurement, the sampling rate was 20 kHz, which is enough to measure small changes in Vm. However, the frame-recording interval of 20 ms in Ca2+ imaging is not fast enough to capture small intracellular dynamics, especially during phase 0 of AP.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
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References


Figure S1: Current-voltage (I-V) relationship of different voltage-gated ionic channels. I-V curve of (A) sodium current ($I_{Na}$) density (B) transient outward potassium current ($I_{to}$) density (C) calcium current ($I_{Ca}$) density (D) rapid rectifier potassium current ($I_{Kr}$) density (E) slow rectifier potassium current ($I_{Ks}$) density (F) inward rectifier potassium current ($I_{K1}$) density (G) funny current ($I_{f}$) density.
Figure S2: Characteristics of DADs in hiPSC-CMs. (A) Action potentials exhibiting DADs without corresponding change in CaT. (Action potential amplitudes and CaT amplitudes were normalized to 1.5 and 1 respectively) (B) The relationship between the percentage of DAD amplitude and percentage of CaT amplitude ($r^2=0.2$, $P=0.01$, Pearson’s correlation test; n=33) (C) Average amplitude of DADs observed in both $V_m$ and CaT (n=33), and only observed in $V_m$ (n=26). (10.4 ± 0.6 mV versus 6.5 ± 0.5 mV; $P < 0.0001$, student t-test (two-tailed))
Figure S3: Patch clamp system synchronized with imaging system. Fluo-4 loaded hiPSC-CMs were continuously bath with extracellular solution. Action potentials are continuously recorded by patch clamp system. Once the calcium transients recording starts, imaging system send synchronization pulses to patch clamp system. Synchronization pulses are halted when the calcium recording stop.
Movie 1 commentary

Live imaging of spontaneously beating hiPSC-CMs. hiPSC-CMs were loaded with Fluo-4. Patch clamp system record action potential and calcium imaging record intracellular calcium transient simultaneously.