Modulating human iPSC-CM electrophysiology for *in vitro* drug screening

control

rise

sustain

decay

waveform

main

I_{Na}

$I_{to,f}$

$I_{Ca,L}$

I_{Kr}

I_{Ks}

I_{K1}

pumps

NKA

NCX

PMCA

SERCA

Birgit Goversen

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Cover design: © 2020 Birgit Goversen. Title: 'The iPSC-derived cardiomyocyte as a modular synthesizer'. Play with your favorite ionic currents in varying shapes and sizes, up- or downscale cardiac proteins, mix the signals and harness the full power of the iPSC-CM.

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Modulating human iPSC-CM electrophysiology for *in vitro* drug screening

Het moduleren van de elektrofysiologie van humane iPSC-afgeleide
cardiomyocyten
voor het dierproefvrij testen van medicijnen
(met een samenvatting in het Nederlands)

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Voor mama

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Each year, the pharmaceutical industry spends billions on drug discovery and development, which is a rather inefficient process with high attrition rates. Between 1953 and 2013, over 450 drugs have been withdrawn from the market worldwide¹. The most common reason for withdrawal is the drug-induced disturbance of cardiac rhythm, which may progress into the rare but potentially lethal Torsade de Pointes (TdP; 'twisting of the points') arrhythmia. Under physiological conditions, cardiac activation starts with spontaneous activity in the sinoatrial node, after which the signal is conducted to the atria, briefly paused in the atrioventricular node, guided through the His bundle, the bundle branches and the Purkinje fibers, finally activating the ventricular muscle. Each part of the conduction system contains specialized cell types with respective unique electrical characteristics.

The ventricular cardiomyocyte activates in response to a change in membrane voltage (potential), which causes an orchestrated response of ions flowing in and out of the cell (action potential (AP), Figure 1A, black trace) that allows the cardiomyocyte to contract. The sudden change in voltage first causes the sodium channels to open and allow an inflow of sodium ions that establish the upstroke of the AP and depolarization of the cell (phase 0). Depolarization persists with rapid closing of sodium channels and opening of potassium channels to allow the efflux of the transient outward potassium current (I_{to}) (phase 1). Consequently, calcium channels open to provide a calcium influx and cause a burst release of calcium from the Sarcoplasmic Reticulum (SR), the intracellular calcium store, through activation of Ryanodine Receptors (RyRs). The elevated calcium levels prolong the activation and enable the cardiomyocyte to contract during the plateau phase (phase 2). Activation ceases with the opening of two specific potassium channels, conducting the rapid delayed rectifier potassium current (I_{Kr}) and the slow delayed rectifier potassium current (I_{Ks}), enabling repolarization (phase 3). Repolarization is completed by the inward rectifier potassium current (I_{K1}) (phase 4), which returns the membrane voltage to the resting membrane potential (RMP). The cardiac action potential is the result of a sum of cooperating ion channels, depending on each other for activation and inactivation. Individual cardiomyocytes are joined together with non-cardiomyocytes to form a syncytium that facilitates action potential propagation throughout both ventricles. All action potentials from the ventricles are reflected in the QRS complex on the electrocardiogram (ECG) which signifies a single heartbeat (Figure 1B, black trace).

The shape and duration of the action potential may change if ion channel function is disturbed, for example as a result of mutations in genes that encode ion channel proteins, trafficking defects of the channels to the membrane, diseases or drugs.

Figure 1: The human cardiac ventricular action potential and ECG.

(A) A single ventricular action potential with the upstroke (phase 0), I_{to} -mediated repolarization (phase 1), plateau phase (phase 2), repolarization (phase 3) and final repolarization (phase 4) at baseline (black trace) and when prolonged (green trace). (B) Schematic representation of the ECG and how it relates to the ventricular action potential; atrial activation (P), ventricular activation (QRS), ventricular repolarization (T) at baseline (black trace) and when prolonged (green trace).

A decrease in repolarizing (potassium) current may not have detrimental consequences due to compensation by other potassium currents (repolarization reserve), but can inhibit repolarization and prolong the cardiac action potential and the QT interval (Figure 1A, B, green traces). An increase in depolarizing (sodium or calcium) current, on the other hand, might subject the cardiomyocyte to early re-activation patterns. When calcium channels remain open after contraction (phase 2) or when sodium channels prematurely open during repolarization (phase 3), early afterdepolarizations (EADs) may occur. When cytosolic calcium levels are elevated during final repolarization (phase 4), delayed afterdepolarizations (DADs) may occur². This abnormal impulse formation might lead to triggered activity, which in turn can act as a substrate for tachycardia.

If part of the conduction of the signal is slowed, by means of a disease or drugs, the electrical signal may propagate through the tissue via different pathways in a heterogeneous fashion (slow and fast). When different pathways of conduction intermingle, the conduction may create a rotating signal that perpetuates in a process named re-entry. Both triggered activity and re-entry may perpetuate into arrhythmias such as TdP³ and consequently the torsadogenic risk of (new) drugs should be quantified during development of those drugs.

The pipeline of drug development consists of distinct phases: identifying a target, synthesizing a new chemical entity (NCE) for the target and testing that NCE for efficacy, sensitivity (to predict whether an NCE is safe) and specificity (to predict whether an NCE is not safe) in preclinical models. The effect of a drug is first evaluated in isolated cells, either in cell lines overexpressing one specific type of ion channel or in single isolated (animal) cardiomyocytes. Ion channel activity and the effect of drugs can be measured by the patch clamp technique, which uses μm wide borosilicate glass pipettes to form a tight connection to a small patch of the membrane of a cell, break the membrane patch to gain intracellular access, and hold, 'clamp', the voltage of a cell in voltage clamp mode. By applying various voltage steps, ion channels can be activated thereby eliciting ion currents which subsequently can be measured. The main current of interest for cardiac safety screening is I_{Kr} , conducted by the Kv11.1 or the human Ether à-go-go Related Gene (hERG) channel, since it facilitates phase 3 repolarization and many drugs target this channel⁴.

After studies in single cells, the drug is tested on a larger scale, first in multicellular preparations using sharp microelectrodes, then in *ex vivo* animal hearts and eventually in living animals. If a compound makes it successfully through these pre-clinical phases, it may continue to clinical trials. These include testing on healthy individuals, small groups of patients for which the drug is dedicated, and finally in large groups. Safety testing for arrhythmic properties of drugs has only been introduced in the 1990s, when the antihistamine terfenadine caused unforeseen arrhythmias in patients with hay fever. Since then, testing drugs for adverse effects on the heart has been embedded in safety pharmacology⁵, which is documented in the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S7B and E14 guidelines. S7B describes the guidelines for safety testing of new compounds in *preclinical* studies in isolated cells, multicellular preparations or animals and E14 describes the guidelines for safety testing of new compounds in *clinical* studies in patients⁶.

Since drug discovery and development are aimed at providing new therapies for humans, logically the use of human material for preclinical testing would be preferable. Unfortunately, human adult cardiomyocytes are difficult to obtain, often only from explanted failing hearts, and do not maintain well in culture. As explained before, currently used alternatives are human cell lines overexpressing one of the cardiac ion channels or animal cardiomyocytes. Using animal cardiomyocytes for assessing drugs aimed at humans does raise questions regarding their applicability, as animals differ significantly from humans in anatomy of the heart (organ size and amount of cells), system physiology (blood needed for the animal size) and cardiac ion channel expression (more or less channels, channels with different characteristics)⁷. The action potential shape and duration clearly reflect those differences. For example: when we compare the modelled ventricular action potentials of rabbits, dogs and humans, the duration seems to differ between ± 200 ms (rabbit), ± 250 ms (dog) and ± 300 ms (human) (Figure 2, black line). Furthermore, both the rabbit and the dog exhibit a clear I_{to} mediated phase 1 repolarization, while phase 1 repolarization is not that pronounced in the human action potential. The plateau phase, essential for calcium dynamics and contraction, is also different between rabbit, dog and human. These differences in action potential duration and shape also suggest that inhibition of one of the cardiac ion channels by a drug has different consequences for action potential characteristics in each species. Indeed, after blocking the I_{Kr} channel for 80% in the model (Figure 2, green line), the action potential prolongs by ± 100 ms in the rabbit and ± 250 ms in the human, but causes afterdepolarizations in the dog. This indicates that repolarization in the canine cardiomyocyte depends more on I_{Kr} than on other potassium currents and therefore seems more sensitive to I_{Kr} block. When a drug is applied with an estimated I_{Kr} blocking capacity of 80%, the drug will have proarrhythmic effects in the dog but not in humans. A drug that proves unsafe in animals thus may prove safe in humans and vice versa. These simulations demonstrate the discrepancies in drug response between species, highlighting the limitations of animal experiments in drug screening.

New possibilities for a source of human cardiac tissue opened up by the development of human embryonic stem cell-derived cardiomyocytes (hESC-CMs). The differentiation of human stem cells into cardiomyocytes generates tissue that contracts spontaneously and provides a human *in vitro* model to study the propagation of electrical signals⁸. hESC-CMs do come with ethical concerns as they are derived from human blastocysts, a precursor of the embryo, which limits widespread investment in research.

Figure 2: Simulation of the ventricular action potential.

Simulations of ventricular action potentials at 1 Hz in rabbit, dog and human under baseline conditions (black trace) and after 80% of I_{Kr} block (green trace). The trace depicted is the last beat after 200 seconds of simulations. Models used: Shannon *et al.*, 2004 (rabbit), Decker *et al.*, 2009 (dog), O'Hara *et al.*, 2011 (human).

A more promising and ethically less sensitive model came with the development of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). iPSCs are somatic cells such as dermal fibroblasts, that are reprogrammed to pluripotent cells, which can subsequently be differentiated towards cardiomyocytes. iPSC-CMs can be derived from anyone and can therefore be patient-specific, while providing a virtually limitless supply of *in vitro* human cardiac tissue⁹. The potential of iPSC-CMs for safety pharmacology has been implemented in a new paradigm to change the field of drug safety testing: the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative. CiPA is designed to humanize safety pharmacology by 1) testing drug effects on ion currents in overexpressing cell lines, 2) including the observed drug effects in computational human ventricular action potential models, 3) screening the drug in iPSC-CMs and 4) translating the results into clinical evaluation by ECG. In this way, safety pharmacology becomes high-throughput, focused on humans, while potentially eliminating the need for animal models¹⁰.

However, the applicability of iPSC-CMs for safety pharmacology has also been disputed over the last years. Many studies have shown that iPSC-CMs, expected to resemble adult human ventricular cardiomyocytes, rather resemble embryonic cardiomyocytes both in structure and in electrical characteristics. Regarding those electrical aspects, iPSC-CMs express channels that conduct the funny current I_f , which is normally only present in sinoatrial cells and contributes to spontaneous activity, and do not contain a well-developed SR which impacts their calcium handling¹¹. Moreover,

low levels of I_{K1} cause an elevated resting membrane potential (~ -60 mV), which contributes even more to the spontaneous activity¹². As in all excitable cells, the ion currents in iPSC-CMs depend on each other for proper activation, which affects drug response. Therefore, the current iPSC-CM fails as a proper model for drug screening and this calls for improvement of the electrophysiology of these cells. In this thesis we will modulate the electrical characteristics of the iPSC-CM by applying molecular, chemical and computational techniques to tweak the ion channel activity in this cell type. We will focus on the expression of I_{K1} , as we postulate that adding a sufficient amount of I_{K1} will push the RMP towards more negative values comparable to the RMP of human adult cardiomyocytes. A stable RMP may counteract the negative effects imposed by I_f and improve the activity of other ion channels, such as sodium or calcium, that depend on a negative RMP. Overall, we expect to improve the quality of iPSC-CM electrophysiology and contribute to a better model for preclinical drug screening *in vitro*.

Therefore we hypothesize in this thesis that *enhancing functional expression of I_{K1} in iPSC-CMs will generate a model that can be fully implemented in drug safety testing, and allows the replacement of currently used animal models with stem cell-derived models.*

In *chapter 2* we describe the role of I_{K1} in cardiomyocytes, provide an overview of iPSC-CM differentiation and illustrate the electrical deficits of iPSC-CMs. In *chapter 3* we virtually inject I_{K1} by means of dynamic clamp and combine the technique with automated patch clamp. In *chapter 4* we determine the threshold of I_{K1} where the cell becomes quiescent to tweak the excitability in iPSC-CMs. In *chapter 5* we overexpress the gene for the Kir2.1 protein, which constitutes the I_{K1} channel, in iPSC-CMs and employ a light-sensitive ion channel to control excitability non-invasively. In *chapter 6* we explore the additive value of iPSC-CMs to precision medicine and describe the electrophysiological read-out of two specific lines of iPSC-CMs coming from patients with either a mutation in desmoplakin (DSP) or in phospholamban (PLN). In *chapter 7* we focus on isoforms of the hERG channel (conducting I_{Kr}) in iPSC-CMs and their implications for drug screening. In *chapter 8* we put all efforts on *in vitro* methods in perspective to the current *in vivo* golden standard, and mirror the feasibility of our approach towards implementation in human drug safety screening.

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Abstract

Preclinical drug screens are not based on human physiology, possibly complicating predictions on cardiotoxicity. Drug screening can be humanized with *in vitro* assays using human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). However, in contrast to adult ventricular cardiomyocytes, iPSC-CMs beat spontaneously due to presence of the pacemaking current I_f and reduced densities of the hyperpolarizing current I_{K1} . In adult cardiomyocytes, I_{K1} finalizes repolarization by stabilizing the resting membrane potential while also maintaining excitability. The reduced I_{K1} density contributes to proarrhythmic traits in iPSC-CMs, which leads to an electrophysiological phenotype that might bias drug response. The proarrhythmic traits can be suppressed by increasing I_{K1} in a balanced manner. We systematically evaluated all studies that report strategies to mature iPSC-CMs and found that only few studies report I_{K1} current densities. Furthermore, these studies did not succeed in establishing sufficient I_{K1} levels as they either added too little or too much I_{K1} . We conclude that reduced densities of I_{K1} remain a major flaw in iPSC-CMs, which hampers their use for *in vitro* drug screening.

Introduction

Drug development is a lengthy process where a potential drug needs to withstand preclinical and clinical phases before it can reach clinical practice. Potential drugs are tested for efficacy and cardiac safety in a series of preclinical models with increasing complexity, including *in vitro* assays using overexpressing cell lines, isolated cardiomyocytes, isolated animal hearts and *in vivo* animal testing¹. The compound may then enter clinical trials to gain approval by the Food and Drug Administration (FDA). Approved drugs may appear safe but prove to be unsafe after reaching clinical practice, causing withdrawal from the market. 45% of withdrawals is caused by cardiovascular complications including Torsade de Pointes (TdP) arrhythmia and sudden cardiac death². These complications are the result of drugs that block cardiac ion channels and modify cardiac rhythm. Mostly blockage occurs of the human Ether-à-go-go-Related Gene (hERG) channel, which facilitates repolarization in cardiomyocytes. This has led to release of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S7b and E14 guidelines, which in combination describe how to test for QT prolonging effects of drugs, and if such prolongation is found, how to test for additional risk using preclinical models. After implementation of the guidelines, no newly introduced drugs have been withdrawn due to TdP incidence³. Unfortunately, excluding all drugs that only block hERG is not satisfactory either, as many safe drugs block hERG and/or prolong QT time without triggering arrhythmias³.

Occurrence of arrhythmia and sudden cardiac death may be prevented by improved prediction of cardiotoxicity in preclinical phases. However, current preclinical drug screens entail *in vitro* cellular systems that are oversimplified, or animal models that do not resemble human electrophysiology regarding action potential (AP) duration and ion channel composition⁴. This limits the sensitivity (the ability to predict whether a compound is safe) and specificity (the ability to predict whether a compound is not safe) of the assay⁵, although performance can be improved by implementation of drug effects on multiple ion channels in computer simulations of cardiac action potentials⁶. Ideally, preclinical drug screens should be completely based on human cardiac electrophysiology. The need for human cardiac models has led to the development of the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative⁷. CiPA aims to establish a new safety testing paradigm that is not exclusively focused on hERG block. Instead, it combines *in vitro* drug screens on overexpression cell lines with computer models to predict drug effects on human action potentials. The *in silico* results are then confirmed with *in vitro* drug screens on human induced pluripotent

stem cell-derived cardiomyocytes (iPSC-CMs). However, reliable predictions of cardiotoxicity require good resemblance between iPSC-CM electrophysiology and adult cardiomyocyte electrophysiology.

Electrophysiology of iPSC-CMs: a sound foundation for safety assays?

Unfortunately, iPSC-CMs do not fully resemble adult ventricular cardiomyocytes, and one of several differences is automaticity of iPSC-CMs. Automaticity causes the cells to beat spontaneously, so they do not require an external depolarizing stimulus to fire an action potential, as adult cardiomyocytes of the working myocardium do. The automaticity is the result of an interplay between depolarizing and repolarizing ion currents. iPSC-CMs are characterized by presence of the funny current (I_f), which is only significantly present in cells in the sinoatrial node of the adult heart⁸ to drive pacemaking. iPSC-CMs also display very low levels of the inward rectifier potassium current (I_{K1}), which stabilizes the resting membrane potential (RMP) in adult cardiomyocytes and is responsible for the final phase of repolarization⁹. In absence of I_{K1} , iPSC-CMs do not achieve a sufficiently negative and stable resting membrane potential (~ -57 to -70 mV instead of -85 mV¹⁰), allowing the depolarizing I_f current to bring the membrane potential over the threshold to trigger an action potential. The combination of a significant density of I_f and a near-complete lack of I_{K1} causes iPSC-CMs to resemble pacemaker cells from the sinoatrial node. A recent mechanistic study confirmed the role of the interaction between I_f and low levels of I_{K1} in pacemaking¹¹. However, in the heart pacemaker cells only represent a small subset of cardiomyocytes, in contrast to ventricular cardiomyocytes, which express more relevant ion channels and therefore are more suitable as a model. The ability of a model to assess drugs targeting multiple ion channels is essential to properly predict drug safety³. Therefore, the presence of I_f and absence of I_{K1} cause proarrhythmic traits in iPSC-CMs that have to be considered carefully¹². In our view, establishing sufficient I_{K1} in iPSC-CMs will be a crucial step to counteract the proarrhythmic traits and increase the usefulness of iPSC-CMs for CiPA. Our view is shared by others, as many methods have been developed to improve iPSC-CM electrophysiology. This review will discuss the role of I_{K1} in adult cardiomyocytes and iPSC-CMs and provide an overview of studies aiming to increase I_{K1} in iPSC-CMs to determine the progress on iPSC-CMs as a model for *in vitro* drug screening.

Cardiac I_{K1} channels are essential facilitators of excitability

The action potential in the ventricular cardiomyocyte requires orchestrated opening and closing of various ion channels. After a depolarizing stimulus arrives, voltage-

gated sodium channels open to allow influx of sodium (phase 0). After a small repolarization, voltage-gated calcium channels open to allow influx of calcium via L-type calcium receptors, and evoke calcium-induced calcium release from the sarcoplasmic reticulum (SR) giving rise to the plateau phase (phase 2). Then the I_{Kr} channels open to facilitate repolarization (phase 3), after which I_{K1} stabilizes the membrane potential (phase 4). iPSC-CMs lack I_{K1} and therefore do not achieve a sufficiently negative membrane potential, leaving the resting membrane potential slightly depolarized (Figure 1A). A depolarized resting membrane potential causes sodium channels, which depend on voltage, to remain inactive¹³. As a result, less functional sodium channels are available for opening when the upstroke in phase 0 starts, which leads to a slower upstroke velocity. iPSC-CMs further express the pacemaker current I_f , which is activated at hyperpolarized voltages (-45 to -60 mV). Altogether, the altered ion channel composition and sequence of activation in iPSC-CMs lead to proarrhythmic traits. The iPSC-CM shows that I_{K1} is not only essential to maintain the resting membrane potential, but also determines the availability of ion channels for the next action potential (Figure 1B).

In adult cardiomyocytes, I_{K1} conducts potassium ions into the cell at membrane potentials that are more negative than the reversal potential of potassium (E_K) which is around -85 mV⁹. At potentials that are less negative than E_K , I_{K1} channels conduct an outward current, but the conductance of the channel decreases sharply with further depolarization. This feature, referred to as rectification, is caused by blocking of the channel's inner pore by magnesium, spermine or spermidine at depolarizing membrane potentials¹⁴. The rectification of I_{K1} channels allows the development of the plateau phase in the cardiac action potential.

The influence of I_{K1} current density can be deduced from several forms of human cardiac disease. Mutations in the KCNJ2 gene, encoding the Kir2.1 protein that constitutes the channel conducting I_{K1} , can lead to either a loss-of-function or a gain-of-function in I_{K1} , and these mutations have been associated with different types of arrhythmias. A loss-of-function mutation in KCNJ2 can result in disrupted Kir2.1 trafficking leading to decreased Kir2.1 channels on the membrane and ultimately a decrease in I_{K1} ¹⁵. The consequences have been also demonstrated in transgenic mice harboring a dominant-negative Kir2.1 channel. In these animals, loss of I_{K1} caused action potential prolongation in isolated cardiomyocytes, and QRS prolongation and QT prolongation in intact hearts¹⁶. A complete knockout of KCNJ2 in mice led to mortality between 8 and 12 hours after birth due to a cleft palate¹⁷. In humans, loss-of-function mutations in KCNJ2 can result in the Andersen-Tawil Syndrome (ATS),

which is an autosomal dominant disease that can present itself with possibly lethal ventricular arrhythmias, periodic paralysis and dysmorphology¹⁸. A gain-of-function in I_{K1} has been associated with Short QT Syndrome and familial atrial fibrillation (AF), in which patients may suffer from aberrant T-waves. Data from computer simulations shows action potential shortening and the possibility of re-entrant arrhythmia¹⁹. Not only a gain-of-function mutation can contribute to arrhythmia. Upregulation of I_{K1} current density in the atria as a result of remodeling has further been associated with atrial fibrillation^{20, 21}. The examples in human disease underline the importance of I_{K1} current density to maintain normal cardiac excitability and rhythm.

Figure 1. (A) Schematic depicting the differences between the action potential waveform of human adult ventricular cardiomyocytes and that of human iPSC-CMs. Numbers indicate the various phases of the action potential. Note the spontaneous depolarization during phase 4 and the slow upstroke velocity of phase 0 in the iPSC-CM. This is in contrast with the action potential in adult CMs that have a stable and more negative resting membrane potential and fast upstroke. **(B)** Illustration of the effects of increasing functional I_{K1} expression in iPSC-CM, derived from simulations done with a model based on data in Jonsson *et al.*, 2012. Action potentials are from an iPSC-CM paced at 1 Hz (top row), and resulting ion currents are displayed below. Each column depicts the result of simulations with differing amounts of functional I_{K1} expression, increasing ten-fold from the left-most to the right-most column. With increasing I_{K1} expression the resting membrane potential of the cell becomes more negative and more stable, which changes the contribution of other ion channels. Sodium current (I_{Na}) during phase 0 increases in peak amplitude, while the peak amplitude of the L-type calcium current ($I_{Ca,L}$) remains relatively similar. The hERG current (I_{Kr}) becomes less active with increasing I_{K1} , changing from a current that is active throughout the whole duration of the action potential to a current that is mainly active during repolarization (phase 3) of the action potential.

Kir2.x proteins shape the cardiac I_{K1} channel

In adult cardiomyocytes, the I_{K1} channel is mostly constituted by tetramers of the Kir2.1 protein, but also Kir2.2 and Kir2.3 may contribute to functional channels. Experiments comparing the Ba^{2+} -sensitivity of various combinations of Kir2.1, 2.2, and 2.3 with that of native I_{K1} of human adult right ventricular cardiomyocytes suggest that human ventricular I_{K1} is generated by a combination of Kir2.1 and Kir2.2²². Hetero-tetramers have altered gating and conductance²³. Kir2.1 is mainly localized in the ventricle and is less abundant in the atria, leading to a reduced I_{K1} current density in the atria²⁴. The expression differences between tissues are mainly controlled by transcriptional regulation of the aforementioned KCNJ2 gene²⁵. So far, three transcription factors have been identified to interact with the KCNJ2 promoter: Sp1, Sp3 and NF-Y. The interaction of these three as a complex, and the interaction of the complex with the promoter is necessary for KCNJ2 transcription²⁵. Transcription of KCNJ2 might be more complex given the wide distribution over tissues and the large variability in expression level within a given tissue or organ, but no other regulators have been reported yet.

The exact role of Kir2.1 during cardiac development remains to be determined

The KCNJ2 gene is conserved throughout vertebrate evolution with functional homology for Kir2.1²⁶. However, the role of Kir2.1 in development of the human heart is not well described, although important lessons may be learned from studies describing the role of Kir2.1 in skeletal myoblast differentiation. Both skeletal muscle and the heart originate from the mesoderm, and in their final differentiated form, skeletal and cardiac myocytes both express high levels of I_{K1} , mostly encoded by Kir2.1¹⁵. Moreover, in skeletal myoblasts, hyperpolarization of the membrane is an early and essential step in differentiation²⁷, which may also be true for cardiac progenitor

cells²⁸. In both cell types, undifferentiated cells have a resting membrane potential of ~ -30 mV, which is gradually hyperpolarized when I_{K1} channels become expressed. As a result of this hyperpolarization, intracellular Ca^{2+} levels increase, activating Ca^{2+} -sensitive transduction pathways (e.g. calcineurin, myogenin, MEF2c), resulting in further commitment to myocyte differentiation. In myoblasts, intracellular Ca^{2+} increases via influx of Ca^{2+} through T-type Ca^{2+} -channels and via store-operated Ca^{2+} entry^{29, 30}, but only L-type Ca^{2+} channels were found in human cardiomyocyte progenitor cells²⁸.

Work in skeletal myoblasts has shown that Kir2.1 channels located in the plasma membrane are not all active, a subset is present but not conductive. Kir2.1 channels are activated by dephosphorylation of a tyrosine residue (242) in myoblasts³¹. This results in an increase in active Kir2.1 channels at the cell membrane and thus conduction of I_{K1} . With regards to forward channel trafficking, a cell surface protein Cdo has recently been identified to recruit Kir2.1 channels to the membrane³². Cdo acts in a complex with p38MAPK which is a regulator of the myoblast differentiation programme, and may influence tyrosine kinase activity. Thus, it seems that early membrane expression and activation of I_{K1} channels depends on Cdo and dephosphorylation of the channel, but if, and to what extent the findings in myoblasts are reflected in other stages of cardiac development or iPS cardiomyocyte differentiation remains to be determined.

Interactions of I_{K1} channels show the involvement in many cellular processes

In contrast to Kir2.1 in developing cardiomyocytes, much more is known on the role of Kir2.1 in adult cardiomyocytes. Proper action potential formation requires a coordinated expression of ion channels, which may be regulated at the level of translation, channel trafficking, phosphorylation, or interaction of ion channels with other proteins, as summarized in Table 1. One of the ion channels Kir2.1 interacts with is $Na_v1.5$, the channel that conducts the cardiac sodium current I_{Na} . $Na_v1.5$ likely forms a macromolecular complex with Kir2.1 in which they reciprocally regulate expression and function^{33, 34}. This macromolecular complex-mediated trafficking is established by an interplay between the cytoskeleton, motor proteins and cell organelles³⁵. The exact composition of the complex is still to be elucidated, but the Synapse Associated Protein (SAP) 97 and α -1-syntrophin have been identified to interact with these ion channels³⁶. SAP97 and Kir2.1 have also been associated with beta-adrenergic receptor 1 (β -AR1) and Protein Kinase A (PKA)³⁷. The interaction between SAP97 and Kir2.1 is likely achieved by a 'Postsynaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein' (PDZ) binding motif, which anchors to other proteins to ultimately physically connect the ion channels. Mutations in the proteins involved in the $Na_v1.5$ -Kir2.1 interaction have been associated with disrupted

$Na_v1.5$ and Kir2.1 assembly and arrhythmogenic consequences³⁵. Kir2.1 has also been associated with calcium handling, with high levels of intracellular calcium correlating to voltage-dependent I_{K1} block³⁸ and upregulation of I_{K1} as a consequence of decreased Calcium-calmodulin dependent protein kinase II (CaMKII) activity³⁹.

The Kir2.1 channel not only interacts with other channels, but also with different lipids and proteins involved in various cellular processes. One of these is membrane-bound Phosphoinositide 4,5-bisphosphate (PIP_2) that increases the open probability to allow full conductance of I_{K1} ⁴⁰. Recent findings suggest that calcium-sensing receptors are activated by phosphatidylinositol 4-kinase (PI-4-K), which in turn increase I_{K1} . In this process, PIP_2 decreases by activation of the receptor and increases by activation of PI-4-K⁴¹. Cholesterol negatively influences Kir2.1 by regulating the open probability⁴². Cholesterol probably regulates Kir2.1 by interfering with PIP_2 binding to the Kir2.1 channel, and has recently been suggested to crosstalk with caveolin-1⁴³. Kir2.1 is further associated with the protein kinase (PK) family, of which PKA and PKC decrease I_{K1} ^{44, 45} and PKB increases I_{K1} by activating phosphatidylinositol-3-phosphate-5-kinase PIKfyve. PKB promotes I_{K1} by increasing Kir2.1 channel abundance on the membrane⁴⁶. The amount of Kir2.1 channels also increases in vascular smooth muscle through interacting with actin-binding protein Filamin A⁴⁷. Interactions with cardiomyocyte specific proteins have been investigated in zebrafish, where I_{K1} is downregulated after knockout of the desmosomal protein plakophilin-2⁴⁸. I_{K1} is also negatively regulated by Kir2.1 trafficking. Retrograde Kir2.1 trafficking can be modulated through the clathrin-mediated endocytosis pathway⁴⁹. Kir2.1 is further regulated on a transcriptional level by microRNA1 (miR-1), which decreases KCNJ2 post-transcriptionally⁵⁰. Furthermore, Kir2.1 appears to be downregulated by miR-26 and miR-101 in atrial fibrillation⁵¹.

iPSC-CM differentiation determines expression of ion channels

Adult somatic cells can be converted to pluripotent stem cells *in vitro* by inducing overexpression of a mix of the transcription factors Klf4, c-Myc, Sox2 and Oct4 or Nanog, Lin-28, Sox2 and Oct4⁵². Pluripotent cells can then be differentiated towards cardiomyocytes by methods which have been established in embryonic stem cell culture, including embryoid body formation, suspension culture, monolayer culture or coculture with feeder cells⁵³.

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* An addendum has been published for this article, explaining scientific misconduct of the corresponding author. A corrigendum has been published for one figure in this article.

Table 1: Interactors of Kir2.1 (I_{K1}) channels

Compound	Group	Kir2.1 ↓ Kir2.1 ↑	Mechanism of action	Cell type
α -1-syntrophin	Protein	X	Part of macromolecular complex mediating reciprocal modulation of $Na_v1.5$ and Kir2.1	CHO + rat ventricular cardiomyocytes ³⁵ & Mouse ventricular myocytes ¹⁰⁶
AKAP 15 and 79	Plasma membrane-bound proteins	X	Colocalize with Kir2.1 and are involved in PKA signaling	<i>Xenopus</i> oocytes + rat ventricular cardiomyocytes ⁴⁴
β 1-AR	Receptor	X	I_{K1} current decreases as a result of β 1-AR activation	Rat ventricular cardiomyocytes ³⁷
CaMKII	Kinase	X	Inhibition of CaMKII leads to upregulation of I_{K1}	Mouse ventricular cardiomyocytes ³⁹
Caveolin-1	Protein	X	Binds to Kir2.1 channel	HEK293 + mice bone marrow derived macrophages ⁴³
Caveolin-3	Protein	X	Protein carrying mutation decreases Kir2.1 surface expression	HEK293 ¹⁰⁷
Cdo	Cell surface protein	X	Forms complex with Kir2.1 during myogenic differentiation to enhance surface expression of Kir2.1	Myoblast C2C12 ³²
Cholesterol	Lipid	X	Influences gating of Kir2.1 channels	CHO ⁴² & <i>Xenopus</i> oocytes ¹⁰⁸
Endothelin-1	Protein	X	PKC-mediated phosphorylation of heteromeric Kir2.1/Kir2.2 channels	Human cardiomyocytes + <i>Xenopus</i> oocytes ¹⁰⁹
Filamin A	Actin-binding protein	X	Interacts with Kir2.1 to increase number of functional channels	Pig arterial smooth muscle cells ⁴⁷
Kir2.2	Ion channel subunit		Forms heterotetramers with Kir2.1	Rabbit ventricular cardiomyocytes ¹¹⁰ & HEK293A ²³

Table 1: Continued

Compound	Group	Kir2.1 ↓ Kir2.1 ↑	Mechanism of action	Cell type
Kir2.3	Ion channel subunit		Forms heterotetramers with Kir2.1	HEK293 ¹¹¹
microRNA-1	microRNA	X	Decreases KCNJ2 expression	Human cardiomyocytes (healthy + CAD), rat ventricular cardiomyocytes + HEK293 ⁵⁰
Na _v 1.5 N-terminal domain	Protein	X	Increases I_{K1} by increasing Kir2.1 expression through reciprocal modulation of Na _v 1.5	Human atrial and rat cardiomyocytes ³⁵
PIKfyve	Protein	X	Increases Kir channel abundance	<i>Xenopus</i> oocytes ⁴⁶
PI-4-K	Kinase	X	Activates calcium sensing receptors, increases I_{K1} current	HEK293T ⁴¹
PIP2	Phospholipid	X	Promotes opening of Kir channel	<i>Xenopus</i> oocytes ⁴⁰
PKA	Protein	X	Inhibits I_{K1} current	<i>Xenopus</i> oocytes + rat ventricular cardiomyocytes ⁴⁴
PKB	Protein	X	Increases Kir channel abundance	<i>Xenopus</i> oocytes ⁴⁶
PKC Beta and Kir2.2		X	Decreases I_{K1} current in Kir2.1 & Kir2.2 heterotetramers	<i>Xenopus</i> oocytes + rat/mouse ventricular cardiomyocytes ¹¹²
Protein Kinase C	Protein	X	Inhibits I_{K1} current	Human atrial cardiomyocytes + <i>Xenopus</i> oocytes ⁴⁵
SAP97	Protein	X	Associates with Kir2.1	Rat ventricular cardiomyocytes ³⁷

All methods use a different approach to spatially organize the cells and yield a different purity of cardiomyocytes.

Next to spatial organization, temporal regulation of pathways involved in cardiogenesis guide differentiation towards cardiomyocytes. Such pathways involve the Wnt and β -catenin pathways and the Bone Morphogenetic Protein (BMP) and Fibroblast Growth Factor (FGF) pathways^{54, 55}. The timing of these pathways can be controlled *in vitro* by adding and removing the involved proteins at defined time points. In addition to temporally and spatially recapitulating cardiogenesis, introducing factors such as retinoic acid, Vascular Endothelial Growth Factor (VEGF), Insulin Growth Factor (IGF)-1 and Activin A has improved the purity of cardiomyocytes^{56, 57}. Nevertheless, current iPSC-CM technology lacks standardized differentiation procedures. This is mainly caused by the different methods to yield a heterogeneous population of cardiomyocytes that cannot be attributed to either atrial, ventricular or nodal cardiomyocytes, display high intra-patient variability, inter-patient variability⁵⁸ and differences between sexes⁵⁹.

The transcriptional profile of iPSC-CMs influences electrophysiology

The expression profile of iPSC-CMs partially resembles that of adult cardiomyocytes. iPSC-CMs express cardiomyocyte markers MYH6, TNNT2⁶⁰, MYL7⁶¹, MHC α -chain and MHC β -chain and MLC⁶². Cardiac transcription factors GATA4, GATA6, MEF2 and NKX2.5 have also been reported in iPSC-CMs⁶⁰. iPSC-CMs further express genes like KCNH2 (I_{Kr}), KCNQ1 (I_{Ks}), SCN5A (I_{Na}), and CACNA1C & CACNA1D (I_{CaL})⁶³ that give rise to channels conducting ion currents. However, gene expression levels do not necessarily translate to functional current densities. For instance, iPSC-CMs express genes encoding for the sodium current, but display a slower sodium upstroke⁶³. iPSC-CMs also express genes coding for the L-type calcium current, but the sarcoplasmic reticulum contains less SERCA2a which impairs SR-mediated calcium handling⁶⁴. Decreased SR-mediated calcium handling means that calcium release in iPSC-CMs mostly depends on entry of extracellular calcium via L-type calcium channels. iPSC-CMs further express decreased levels of RYR2 (ryanodine receptor 2), and NCX1 (sodium-calcium exchanger)⁶³. As has been mentioned before, iPSC-CMs present high transcriptional levels for HCN4 (I_h) and lack transcription of KCNJ2 (I_{K1})¹². hiPSC-CMs further express high levels of Kv1.4 and low levels of KChIP2, both involved in the transient outward potassium current (I_{to}). This corresponds to slower reactivation of I_{to} in hiPSC-CMs⁶⁵.

iPSC-CMs have power in predicting hERG block-mediated Torsadogenic risk

hiPSC-CMs have been used in multiple studies to screen for drug-induced Torsadogenic potential. Although variable responses can be found between cell lines and facilities, iPSC-CMs show action potential duration (APD) prolongation and/or field potential duration (FPD) prolongation with high specificity and sensitivity^{66, 67, 68}. Drug safety assays using iPSC-CMs work best with drugs blocking the hERG current, which is also most frequently blocked in TdP⁶⁹. However, drugs affecting other ion currents are also involved in cardiotoxicity, which can be observed from drug screens on iPSC-CMs as well. However, iPSC-CMs do not detect risks for late sodium⁶⁸ and may give false predictions for $Na_v1.5$ inhibition⁶⁶. An earlier *in silico* study comparing the effects of ion current block in a hiPSC-CM model versus an adult ventricular cardiomyocyte model found that hiPSC-CM are more sensitive to I_{CaL} and I_{K1} block⁷⁰. A recent study overexpressed KCNJ2 in iPSC-CMs and compared drug responses between wild type iPSC-CMs and iPSC-CMs overexpressing KCNJ2 and I_{K1} . Both cell types were subjected to various K^+ channel blockers and the drug response in overexpressing iPSC-CMs resembled adult ventricular cardiomyocyte drug response, whereas wild type iPSC-CMs did not. These results were also confirmed by *in silico* experiments⁷¹.

Making the right comparison: the human fetal cardiomyocyte does not beat spontaneously

The electrophysiological phenotype of human Embryonic Stem Cell-derived cardiomyocytes (hESC-CMs) or iPSC-CMs is often compared to that of the human fetal cardiomyocyte. However, literature on the electrophysiology of the human fetal heart suggests differently. Several groups have published data on the electrophysiology of the late embryonic human heart, ranging in age between 7 and 14 weeks in utero⁷².⁷³. All studies found that the atria and ventricles of those hearts are intrinsically quiescent, *i.e.* do not beat spontaneously, but are triggered by the sinoatrial node. The reason for this is also clear, since human fetal cardiomyocytes have a stable and negative resting membrane potential of ~ -80 mV. As a result, upstroke velocity of the action potential is fast (~ 120 V/s) and the ventricular cardiomyocytes show a clear plateau phase comparable to adult cardiomyocytes.

The electrophysiological phenotype of iPSC-CM does not resemble that of a late embryonic or fetal heart, but may instead resemble that of the first cardiomyocytes of the early human embryo, at the stage of heart tube formation. A direct comparison has been made for mouse, confirming similarity between mESC-CMs and cardiomyocytes from the heart tube⁷⁴. Unfortunately, there are no publications on the electrophysiology

of the human heart tube. As a result of the electrophysiological resemblance to early embryonic cardiomyocytes, iPSC-CMs are often labelled as 'immature'. Strategies that aim to increase the potential of iPSC-CMs and to approximate the fully differentiated state have been labelled as maturation strategies. Care should be taken to describe and assess maturation carefully, as studies often only focus on a limited set of iPSC-CM properties, whereas it would be best to take a broader perspective, including structural, functional, metabolic and molecular maturation⁷⁵.

Optimizing iPSC-CM culture conditions

The overall electrical and structural immaturity of iPSC-CMs have evoked a plethora of methods to improve these cells: changing culture surfaces⁷⁶, applying biochemical stress by electrical stimulation⁷⁶, co-cultures with non-cardiomyocytes⁷⁷, overexpression, prolonged cell culture⁷⁸, and 3D engineered heart tissues (EHTs)⁷⁹.⁸⁰. None of these methods have been reported to be specific for enhancing I_{K1} . One method that can be applied to specifically enhance I_{K1} during a patch clamp experiment is the dynamic clamp method⁸¹.

In dynamic clamping, the membrane potential of a single cardiomyocyte is measured, which is subsequently used to calculate in real-time an appropriate I_{K1} current that is in turn injected into the cell, effectively creating a hybrid composed of a real cell and an ion channel model⁸². Injection of I_{K1} into an iPSC-CM will influence AP shape and duration (Figure 1B). The dynamic clamp method allows precise control of the I_{K1} current density that is injected into the cell and thereby allows modification of APs from iPSC-CMs. Currently, several dynamic clamp implementations have been published. In their elegant study, Meijer van Putten and co-workers made direct comparisons between the effects on iPSC-CM action potentials of three different I_{K1} models. Used were the "Kir2.1" model derived from I_{K1} in HEK293 cells⁸³, the "TNNP" model which is based on I_{K1} in ventricular myocytes in physiological and pathological states⁸⁴. Injected I_{K1} from both these models lead to APs with a more pronounced sodium upstroke and plateau phase. Dynamic clamp using I_{K1} from the third model, the "Bett" model⁸⁵, yielded considerably shorter action potentials, due to the low inward rectification in this model which results in suppression of the plateau.

Effects of optimization strategies on I_{K1} function

Quantifying the effect of a maturation strategy in iPSC-CMs ideally includes effects on the transcription of KCNJ2, the expression of Kir2.1 and/or I_{K1} current densities. If a strategy results in more mature I_{K1} function, this would mean an increase in I_{K1}

current density compared to control iPSC-CMs. Increased KCNJ2 or Kir2.1 levels do not necessarily result in a functional increase in I_{K1} , since Kir2.1 can be subjected to post-translational modifications and trafficking.

We screened all studies that aim to mature iPSC-CMs or ESC-CMs for I_{K1} current densities. Out of approximately 60 studies, only 6 report I_{K1} current densities (summarized in Table 2). The information provided about I_{K1} function in these studies is limited. For instance, little is known about the rectification properties of the currents, which depends on Kir2.1 isoforms expressed, but also on the relative abundance of *e.g.* polyamines and intracellular Mg^{2+} . In order to improve our understanding of I_{K1} function in iPSC-CMs, it would be valuable to address this in future studies. The most important missing parameter is conductance density (G_{max} , *i.e.* the maximal conductance of the inward component, normalized to membrane capacitance) which is a good measure of the amount of functional I_{K1} channels in the plasma membrane. Calculation of rectification factor¹⁵ allows for an estimation of the functional contribution of the Kir2.1 isoforms to recorded I_{K1} current. Dynamic clamp studies have not been included in Table 2 since I_{K1} is injected in those studies and therefore the current density is given and not measured. Current densities for I_{K1} at -120 mV in human adult cardiomyocytes are ~ -11.5 pA/pF⁸⁶.

Table 2: Maturation studies in stem cell-derived cardiomyocytes that increase I_{K1}

Author, year	Cell type	Strategy	I_{K1} density	Reversal potential based on patch clamp solutions
Sartiani <i>et al.</i> , 2007	hESC H1	Long-term culture	At -90 mV: 3.4 ± 1.3 pA/pF	-87.0 mV
Kim <i>et al.</i> , 2010	hESC-CM	Co-culture with cardiomyocyte spheroids	At -100 mV: -1.67 ± 0.50 pA/pF	-85.0 mV
Ma <i>et al.</i> , 2011	hiPSC-CM	N/A	At -123 mV: -2.3 ± 0.6 pA/pF At -35 mV: 1.0 ± 0.2 pA/pF	-88.8 mV
Doss <i>et al.</i> , 2012	hiPSC-CM	Long-term culture	At -100 mV: -3.49 ± 0.91 pA/pF	-82.6 mV

Table 2: Continued

Author, year	Cell type	Strategy	I_{K1} density	Reversal potential based on patch clamp solutions
Nunes <i>et al.</i> , 2013	hPSC-CM	Embedded in 3D collagen-I-gel with mixture of supporting non-cardiomyocytes + pacing at 6 Hz	At -100 mV: -1.53 ± 0.25 pA/pF	-90.7 mV
Vaidyanathan <i>et al.</i> , 2016	hiPSC-CM	Kir2.1 adenovirus	At -120 mV: -29.8 ± 6.9 pA/pF	-84.8 mV

Out of the six optimization studies, long-term culture has been employed in three studies. In the first study, hESC-CMs were kept in culture from 24 to 108 days after which the current densities for I_{K1} were compared. I_{K1} current density increased from 0.6 pA/pF at day 24 to 3.4 pA/pF at day 108⁸⁷. However, the voltage-current relationship for I_{K1} showed little rectification, indicating altered kinetics for I_{K1} . In the second study hESC-CM cardiomyocyte spheroids (*i.e.* embryoid bodies containing only cardiomyocytes) were studied up to 60 days in culture and current densities increased from -0.41 pA/pF to -1.67 pA/pF at day 60⁸⁸. In the third study, iPSC-CMs were kept in culture up to 121 days after which I_{K1} current densities were compared at three different time points. Current densities initially increased from 0.79 pA/pF at day 29 to -3.49 pA/pF at day 74. Later, current density decreased again to -2.17 pA/pF at day 121⁸⁹. These results suggest a decrease of current density after long-term culture, which is opposite to the findings in hESC-CMs. I_{K1} current densities have also been reported in iPSC-CMs where no optimization strategy was applied, with an average of 2.3 pA/pF⁹⁰. In one other study a 3D tissue was created where iPSC-CMs were dispersed in a collagen I hydrogel with non-cardiomyocytes. The so-called 'biowires' were subjected to different pacing regimens and I_{K1} density increased to -1.53 pA/pF. However, KCNJ2 expression decreased in the paced cultures⁹¹. Recently, I_{K1} channels were overexpressed in iPSC-CMs using adenoviral vectors, where I_{K1} current density increased to -30 pA/pF⁹². However, these values were measured 3 or 4 days after transfection and were not monitored on the long term. Furthermore, the iPSC-CMs became quiescent and needed electrical stimuli to fire action potentials. Quiescence indicates the loss of spontaneous (proarrhythmic) activity as a result of I_{K1} , which may have negative effects on maturation of the contractile function of iPSC-CMs. This can be counteracted by overexpression of channelrhodopsin in order to

achieve light-based pacing of cultures, as has been demonstrated in cardiomyocyte monolayers and heart tissue^{93, 94}.

Cardiomyocyte dissociation and culture conditions may reduce functional I_{K1} expression

As outlined above, I_{K1} current densities in iPSC-CMs are generally low, even after applying maturation strategies, and therefore not comparable with the current densities observed in adult cardiomyocytes. This may be explained by insufficient progression of differentiation, but also the *in vitro* environment to which the cardiomyocytes are exposed is likely to limit maturity and reduce functional I_{K1} expression. I_{K1} current densities can for instance be influenced by methods used to obtain single cardiomyocytes from animal hearts. This was demonstrated in neonatal mouse cardiomyocytes that were isolated using identical enzyme solution but by two different methods: Langendorff perfusion and chunk digestion. Cardiomyocytes obtained via Langendorff perfusion method had much higher I_{K1} densities than those obtained via chunk digestion, -45.5 ± 8.0 pA/pF vs. -9.2 ± 1.8 pA/pF respectively (at -110 mV), which was accompanied by a strong depolarization of the resting membrane potential from -80.6 ± 1.1 mV to -60.5 ± 2.6 mV⁹⁵. The I-V curve for I_{K1} in cardiomyocytes obtained by chunk digestion also displayed decreased inward rectification, indicating that the digestion method not only influences I_{K1} current densities, but also the kinetics of the current.

Clearly, conditions in culture differ from those experienced by cardiomyocytes *in vivo*. It is remarkable that cultured human fetal ventricular or atrial cardiomyocytes display strongly depolarized resting membrane potentials (~ -40 mV⁹⁶) compared to the resting membrane potentials that were recorded *in situ* (~ -80 mV, see section 'Making the right comparison...beat spontaneously'). Though not tested experimentally, a possible explanation for the depolarization of primary human fetal cardiomyocytes with prolonged culture might be a decrease in functional I_{K1} expression, as was reported for animal cardiomyocytes. For example, in rat cardiomyocytes, I_{K1} current density decreased approximately 50% after seven days in culture⁹⁷. Decreases in I_{K1} current density have also been reported in rabbit cardiomyocytes, varying from a rapid drop in current density of 54% after 24 hours and no further increase or decrease for six days⁹⁸, to a gradual decrease of 83% in current density after four days in culture⁹⁹. In adult canine cardiomyocytes, I_{K1} conductance density decreased approximately 30% between one and seven days in culture, with no significant change in resting membrane potential¹⁰⁰.

Cell culture not only influences ion channel expression of cardiomyocytes, but also the morphology of the cell. Cardiomyocytes become shorter, rounder and lose T-tubules¹⁰¹, which are invaginations in the adult cardiomyocyte membrane that cooperate with ion channels and receptors to promote excitation-contraction coupling¹⁰². The reduction of T-tubules in cardiomyocytes has been associated with a reduction of I_{K1} current density⁹⁹. Several studies have aimed to restore T-tubules in culture in neonatal rat cardiomyocytes (NRCMs). In one study, NRCMs were dispersed in a 3D "tissue patch" mimicking the human epicard. After three weeks in culture, the cardiomyocytes showed T-tubulation and co-localization of L-type calcium channels and ryanodine receptors indicating improved excitation-contraction coupling¹⁰³. Indeed, calcium transients and action potential propagation velocity improved, resembling the properties of the adult myocardium. Improved electrophysiological characteristics were confirmed in another study using NRCMs in a 3D graft. Cardiomyocytes showed increased T-tubulation and improved calcium handling after electrical field stimulation¹⁰⁴. Since iPSC-CMs lack T-tubules¹⁰⁵, introducing T-tubules in culture might prove to benefit I_{K1} current densities.

Conclusion

Drug attrition rates due to cardiotoxicity are high and may be decreased by proper preclinical drug screens. The current preclinical drug screens do not sufficiently recapitulate human electrophysiology. Therefore a new *in vitro* preclinical drug screen has been proposed in CiPA using iPSC-CMs. However, iPSC-CMs display automaticity and proarrhythmic behavior due to presence of I_f and low levels of I_{K1} . They further express a slower sodium upstroke and have impaired SR-mediated calcium handling. Increasing I_{K1} could stabilize the resting membrane potential and attenuate the proarrhythmic traits of iPSC-CMs. Until now, most studies investigating strategies to increase I_{K1} do not report on I_{K1} or do not produce iPSC-CMs with adult I_{K1} expression levels. This can partly be explained by cell culture, which influences I_{K1} densities greatly. Establishing I_{K1} in iPSC-CMs is essential to achieve an *in vitro* model for the human heart and currently hampers fulfilment of the potential of *in vitro* drug screening initiatives like CiPA. iPSC-CMs show high specificity and sensitivity for hERG inhibition in drug screens, but less sensitivity for compounds blocking other cardiac ion currents. Adding I_{K1} to iPSC-CMs might increase this sensitivity, as has been demonstrated in a recent study. Future studies aimed at maturing iPSC-CMs should compare wild-type iPSC-CMs to 'matured' iPSC-CMs, perform drug screens and focus more on I_{K1} electrophysiology.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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A commentary on:

Reciprocal modulation of I_{K1} - I_{Na} extends excitability in cardiac ventricular cells

by Varghese, A. *Frontiers in Physiology* 7:542, 1–10 (2016).

We read with great interest the excellent paper by Varghese who describes an *in silico* approach to study the consequences of reciprocal regulation of expression of the sodium current (I_{Na}) and the inward rectifier potassium current (I_{K1}) in the ventricle for cardiac excitability and conduction¹. The *in silico* approach follows the experimental results obtained by Milstein *et al.*² who demonstrated functional co-regulation of the sodium and inward rectifier currents, and their underlying channel proteins $Na_v1.5$ and $Kir2.1$ respectively, and electrophysiological consequences upon overexpression in rodent cardiomyocytes with respect to action potential duration and re-entry based arrhythmia propensity. Varghese adapted the guinea pig ventricular cardiomyocyte model, developed by Noble and colleagues³, that is extrapolated to simulations for one dimensional cardiac fibres, in which the fibre is represented as a linear cable model. Varghese changed either the conductance for I_{Na} and I_{K1} individually or in tandem, to assess their influence on the excitability of mammalian ventricular cardiomyocytes. One of the most interesting findings in this paper is the dominance of I_{K1} over I_{Na} in regulation of cardiac excitability, which yields important questions about the significance of the inward rectifier in this process. This commentary will put these results in a broader context in order to provide a framework for future research questions.

The experimental data of Milstein *et al.*² point to the existence of a macromolecular complex in which the SAP97 protein may have a major role in reciprocal regulation of expression of $Na_v1.5$ and $Kir2.1$ proteins, since both channels present binding motifs for SAP97. Milstein and colleagues stress that the cell biological principles underlying reciprocal expression at the sarcolemma are only partly resolved and propose a role for ion channel trafficking in the process. Indeed, $Na_v1.5$ promotes $Kir2.1$ protein to be presented at the cell surface, and it decreases $Kir2.1$ internalization. Whether and to which extent $Kir2.1$ affects $Na_v1.5$ protein trafficking still needs to be resolved. Furthermore, a number of additional proteins are candidate in establishing $Na_v1.5$ - $Kir2.1$ macromolecular complexes at the plasma membrane as well as intracellularly⁴. Finally, the subcellular localization of $Kir2.1$ and $Na_v1.5$, e.g. intercalated disc vs. lateral membranes, may very well depend on the nature of the macromolecular complex.

When location specific complexes exist, we may predict that these respond differently to disease causing factors and thereby change anisotropy. For now, the field has to elucidate the composition of (additional) molecular complexes from native cell types and more importantly, gain knowledge on $Na_v1.5$ and Kir2.1 stoichiometry in such complexes and determine whether variations in stoichiometry between complex types exist, and if so, decipher its significance.

In contrast to $Na_v1.5$, which is predominantly present in the heart, Kir2.1 channels are expressed in many excitable tissue types, like skeletal and smooth muscle, neuronal cells, but also in non-excitable tissues (reviewed in De Boer *et al.*⁵). This sets the stage for efforts to explore potential reciprocal modulation of Kir2.1 and various sodium channel subtypes in non-cardiac tissues, a hypothesis already put forward in the field of epilepsy⁶. Not only does Kir2.1 protein distribution differ between tissue types, there is also variation within the heart. For example, atria and Purkinje fibres express less Kir2.1 channels than ventricles. This spatial variation also holds true for development and disease. Transcriptional differences have been seen in development and upregulation of Kir2.1 has been associated with progression of atrial fibrillation⁵. All these expressional differences likely play a role in action potential formation and propagation, and it may be clear that a complete set of *in silico* models, representing different cardiac tissue types and developmental stages may be required to fully appreciate the functional roles of reciprocal modulation. Currently, both the guinea pig ventricular cardiomyocyte model as the linear cable model do not inhabit these dynamic features and by definition cannot provide clues on anisotropy.

The findings by Varghese might be of importance to the field of induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs). iPSC-CMs are of interest for tissue engineering and *in vitro* drug screening purposes, but their electrical immaturity has to be considered carefully⁷. Aside from an ill-developed sarcoplasmic reticulum, their main drawback is spontaneous beating activity due to a lack of I_{K1} ⁷. Restoration of Kir2.1 and therefore I_{K1} might electrically mature these iPSC-CMs. Since the absence of I_{K1} is closely linked to very low transcription levels of the Kir2.1 producing gene (KCNJ2) in this cell type, it appears obligatory to resolve KCNJ2 promoter regulation first and subsequently use gene specific transcription factors or their upstream regulatory pathways to increase Kir2.1 mRNA expression levels. Once Kir2.1 protein expression level is elevated, the reciprocal modulation of sodium and inward rectifier channels might provide additional means to enhance Kir2.1 and thus I_{K1} in iPSC-CMs. The study by Varghese shows that the interaction between sodium channel and the inward rectifier yields functional implications that cannot be ignored.

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Author contributions

BG, TdB and MvdH wrote the submitted commentary on an original contribution by Anthony Varghese.

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Abstract

An important aspect of the CiPA (Comprehensive *in vitro* Proarrhythmia Assay) proposal is the use of human stem cell-derived cardiomyocytes and the confirmation of their predictive power in drug safety assays. The benefits of this cell source are clear; drugs can be tested *in vitro* on human cardiomyocytes, with patient-specific genotypes if needed, and differentiation efficiencies are generally excellent, resulting in a virtually limitless supply of cardiomyocytes. There are, however, several challenges that will have to be surmounted before successful establishment of hSC-CMs as an all-round predictive model for drug safety assays. An important factor is the relative electrophysiological immaturity of hSC-CMs, which limits arrhythmic responses to unsafe drugs that are pro-arrhythmic in humans. Potentially, immaturity may be improved functionally by creation of hybrid models, in which the dynamic clamp technique joins simulations of lacking cardiac ion channels (*e.g.* I_{K1}) with hSC-CMs in real-time during patch clamp experiments. This approach has been used successfully in manual patch clamp experiments, but throughput is low. In this study, we combined dynamic clamp with automated patch clamp of iPSC-CMs in current clamp mode, and demonstrate that I_{K1} conductance can be added to iPSC-CMs on an automated patch clamp platform, resulting in an improved electrophysiological maturity.

Introduction

The Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative aims to find new means of predicting the proarrhythmic risk of newly developed drugs¹, which do not rely exclusively on hERG block, and not on QT prolongation at all. Key aspects are to include results of computer simulations of drug effects on heart rhythm and *in vitro* assays using human stem cell-derived cardiomyocytes (hSC-CMs).

Characterization of the hSC-CM electrophysiological phenotype has so far shown that these CMs express most, but not all cardiac ion channels, which has implications for their use in safety pharmacological assays^{2,3}. Importantly, the inward rectifying potassium channel, I_{K1} ⁴, that is highly expressed in adult CMs is not, or hardly, expressed by hSC-CMs^{5,6}, while the pacemaker current I_f is expressed consistently. As a result, hSC-CMs display a pacemaker-like phenotype, with a depolarized and unstable resting membrane potential, resulting in spontaneous triggering of action potentials. This is an important issue, as the action potential waveform affects the activity and availability of many cardiac ion channels, as these are voltage-sensitive and rely on a negative resting membrane potential between beats to recover from inactivation after an action potential. For example, in hSC-CMs, sodium channel availability during the action potential is minimal, even though the channels are expressed in sufficient levels. As a consequence, we found in a previous study that, except for drugs blocking the hERG channel, none of the tested drugs that are known to be proarrhythmic in adult cardiomyocytes triggered arrhythmias (early-after-depolarizations) in hSC-CMs². More recent studies have found that iPSC-CMs are not sufficiently mature to detect risks associated with inhibition of the late sodium current⁷, or peak sodium current⁸. Interestingly, adenovirus mediated overexpression of I_{K1} channels in iPSC-CM was demonstrated to improve drug responses⁹.

Several approaches are being adopted to improve the electrophysiological phenotype, for instance overexpression of the I_{K1} channel, which has shown promising results¹⁰. Another, more controllable approach is to use dynamic clamp to add simulated I_{K1} channels to hSC-CMs^{11,12}. The essence of dynamic clamp is that a hybrid model is created by connecting a real cell with a computer simulation of (parts of) a cell. For this to work, one needs a computer simulation that is running in real-time - simultaneously with the experiment on the real cell - so there is an instantaneous interaction between the real cell and the simulation. This works well, and has been described by several groups that applied it in manual patch clamp experiments^{13,14}. Addition of simulated or overexpressed I_{K1} channels results in a stable, more negative

resting membrane potential, increased action potential amplitude and upstroke velocity, thereby bringing the action potential waveform much closer to that of adult human ventricular cardiomyocytes. The expectation is that this approach will result in a more reliable prediction of drug effects, but this hypothesis has yet to be studied systematically⁶.

When considering the use of dynamic clamp in safety pharmacology, the low throughput and complex nature of manual patch clamp in combination with dynamic clamp is problematic. Additionally, as noted in the literature¹⁴, current implementations require the simultaneous use of two computers that both require user interaction during the experiment, which is not very practical. In this study, we have developed a remote-controlled dynamic clamp system with the purpose to couple and integrate it with automated patch clamp devices, in order to increase throughput and develop new predictive assays using hSC-CMs that are in line with the aims of the CiPA initiative. In this study, we demonstrate its application by creating hybrid human cardiomyocyte models by the addition of virtual I_{K1} current to single, suspended human iPSC-CMs, and recording action potentials on an automated patch clamp device.

Material and Methods

iPSC-CM culture and dissociation

Differentiated iPSC-derived cardiomyocytes (Cor.4U, kindly provided by Axiogenesis AG, Germany and Cellartis Cardiomyocytes, kindly provided by Takara Bio Europe AB, Sweden) were cultured according to the suppliers' instructions. The cells were dissociated by incubating them for 15-30 min in TrypLE (Gibco) until detached from the surface of the culture flask, and then kept at 4°C for 30 min before pipetting them to individualize the cells.

Automated patch clamp electrophysiology

Recordings from single iPSC-CMs were done using a Nanion Patchliner automated patch clamp device at 20°C and standard medium resistance NPC-16 chips. After catching an iPSC-CM, obtaining a gigaseal and breaking into whole cell configuration, several experiments were performed. From a holding potential of -100 mV, current-voltage recordings were made using voltage steps from -80 mV to 40 mV for 20 ms increasing in 10 mV steps at 2 s intervals (sodium ion currents), and from -40 mV to 40 mV for 200 ms increasing in 10 mV steps at 5 s intervals (calcium ion currents), with a 100 ms pre-pulse to -40 mV to inactivate sodium ion currents. I_{K1} currents were

recorded from a holding potential of -40 mV, with voltage steps from -120 to +30 mV for 1200 ms increasing in 10 mV steps. I_{K1} was blocked by adding 10 μ M Ba^{2+} , from these recordings we calculated the Ba^{2+} -sensitive steady-state current and report these in I-V diagrams.

Next, the recording mode was switched to current clamp and the effect of adding simulated I_{K1} conductance to the iPSC-CM using dynamic clamp was tested. To this end, a current stimulus was optimized for each cell individually to reliably induce action potentials (APs) at a rate of 0.5 Hz. The stimulus was 1 ms long and ranged from 0.6 to 3 nA. The same stimulus was used to trigger APs while exposing the cells to increasing simulated I_{K1} conductance. If the conductance was set too low, no effect on the AP was detectable, if set too high, no AP could be induced. The simulated I_{K1} conductance range varied considerably between individual cells, 200 pS/pF to 2000 pS/pF were used across cells. All experiments were done using extracellular solution containing (in mmol/L) 140 NaCl, 10 HEPES, 5 Glucose, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.4 (NaOH), 298 mOsm, and intracellular solution containing 110 KF, 10 KCl, 10 NaCl, 10 HEPES, 10 EGTA, pH 7.2 (KOH), 285 mOsm. In some experiments, potassium salts were replaced by cesium salts to expose calcium ion currents otherwise obscured by potassium ion currents. Since potassium currents turned out to be negligible, no difference referring to (real) potassium conductance was observed between those recordings.

Dynamic clamp system

Experiments were done using a dynamic clamp system developed in house at UMC Utrecht, Utrecht, The Netherlands. The system runs on the Labview RT operating system, and simulates I_{K1} current in response to membrane potential measured from the iPSC-CM. Real-time simulation of the I_{K1} current was done using the model by Ishihara *et al.*¹⁵, and simulations were done at 20 kHz, on four independent channels, allowing simultaneous dynamic clamp of four iPSC-CMs. We have included as supplementary information the used I_{K1} model (Supplementary Data Sheet 1, online), a diagram explaining our dynamic clamp implementation (Supplementary Figure 1) and a flowchart describing the various steps taken during the dynamic clamp experiments (Supplementary Figure 2). The dynamic clamp system is coupled to the HEKA EPC-10 Quattro amplifier that is part of the Patchliner setup (see Figure 1). The connections couple the membrane potential of each iPSC-CM with each I_{K1} simulation channel and return the computed I_{K1} current to the iPSC-CM via the external stimulus input of the EPC-10 amplifier. Remote control is achieved via additional couplings that allow bi-directional communication between the dynamic clamp system and the Patchliner setup.

Figure 1: Block diagram showing connections between Patchliner, patch clamp amplifier and dynamic clamp system. For a more detailed depiction of the dynamic clamp system we refer to Supplementary Figure 1.

Table 1: iPSC-CMs can be captured efficiently using automated patch clamp and display Na^+ and Ca^{2+} currents

Capture rate (%)	I_{Na} larger than 50 pA (%)	$I_{Ca,L}$ larger than 50 pA (%)
58 (28/48)	71 (20/28)	68 (19/28)

Six experiments were performed using a total of 3 chips, therefore 48 potential sites on the chip were available and 28 Cellartis iPSC-CMs were captured (with seal resistance > 150 MΩ) resulting in a success rate of 58% for capture.

Table 2: Average electrophysiological parameters iPSC-CMs

R_{Seal} (M Ω)	C_m (pF)	R_s (M Ω)	I_{Na} at -30 mV (nA)	$I_{\text{Ca,L}}$ at 10 mV (pA)
976 ± 144 (28)	37 ± 6 (28)	6.0 ± 0.9 (28)	-5.4 ± 1.5 (7)	-157 ± 24 (18)

Shown are values for seal resistance (R_{Seal}), cell capacitance (C_m) and series resistance (R_s) for Cellartis Cardiomyocytes captured with seal resistance > 150 M Ω . Na^+ current at -30 mV and Ca^{2+} current at 10 mV is also shown. Number of cells shown in brackets. Note that the average current is taken from the I-V curves and not all cells which had a detectable Na^+ current were used for the I-V analysis.

These are used to continuously read dynamic clamp system status and set model parameters (e.g. I_{K1} conductance, membrane capacitance or external K^+ ion concentration) when needed. Converting a model parameter to a digital command was done using macros in Patchmaster. Because of this tight integration with the APC and its software, no direct user interaction with the dynamic clamp setup is necessary. The dynamic clamp system is completely controlled from within the HEKA PatchMaster or the Patchliner PatchControlHT software used to run experiments, and can be set automatically using the programming features in these software programs (i.e. Protocol Editor in PatchMaster or Tree Editor in PatchControlHT).

Statistics

All results are presented as mean $\pm$ standard error of the mean (SEM). Differences in mean outcomes were tested using a One-way ANOVA followed by Tukey's multiple comparisons test, p-values smaller than 0.05 were considered significant.

Results

Na^+ and Ca^{2+} currents in iPSC-CMs

Single iPSC-CMs dissociated from iPSC-CM cultures were loaded in the automated patch clamp device and studied in voltage clamp mode to determine presence of Na^+ and Ca^{2+} currents. Capture of the iPSC-CM in the patch clamp chip was efficient, with appropriate seals in 58% of captured cells (see Table 1). After obtaining whole cell configuration, Na^+ and Ca^{2+} currents could be recorded in ~70% of iPSC-CMs (see Table 2). Current-voltage relations of both currents were similar to those reported for iPSC-CMs in manual patch clamp experiments¹⁶, showing maximal peak current amplitudes at -30 mV for Na^+ (Figures 2A, B) and 10 mV for Ca^{2+} currents (Figures 2C, D). The Ca^{2+} currents could be blocked by nifedipine with an IC_{50} of 252 ± 186 nM, confirming we recorded current conducted by L-type Ca^{2+} channels (Figures 2E, F).

Figure 2: Typical recordings from hiPSC Cellartis Cardiomyocytes recorded on the Patchliner. (A) Na^+ currents in response to increasing voltage steps. (B) Corresponding current-voltage plot for an average of 7 cells. A Boltzmann fit revealed a V_{half} of activation of -46 mV. (C) Ca^{2+} currents in response to increasing voltage steps. (D) Corresponding current-voltage plot for an average of 18 cells. A Boltzmann fit revealed a V_{half} of activation of -5.8 mV. (E) Raw traces of Ca^{2+} current in control conditions (black) and after inhibition by increasing concentrations of nifedipine (blue). (F) The concentration response curve (normalized to maximum block) for nifedipine for an average of 5 cells. The average concentration response curve was fitted with a standard Hill-equation which revealed an $\text{IC}_{50} = 252 \pm 186 \text{ nM}$ (n=5).

I_{K1} currents in iPSC-CMs

Functionality of I_{K1} currents was studied in single dissociated iPSC-CMs that were loaded in the automated patch clamp device. Voltage clamp studies in control solution, and in presence of $10\ \mu\text{M}$ Ba^{2+} to block I_{K1} currents, showed that we could record Ba^{2+} -sensitive currents with some resemblance of I_{K1} in 7 out of 12 cells. Examples of cells showing Ba^{2+} -sensitive and insensitive currents can be found in Figures 3A, C, respectively. However, current densities were low, and both the reversal potential (which was less negative than expected) and the rectification of the currents were not as observed in adult cardiomyocytes (Figures 3B, D).

Figure 3: I_{K1} recorded in Cor.4U cells on the Patchliner.

(A) Example of a cell with I_{K1} , shown are responses to a voltage step protocol to -120mV for 1200 ms from a holding potential of -40 mV in control conditions (black) and in the presence of $10\ \mu\text{M}$ BaCl_2 (green). (B) Average current-voltage relationship of the Ba^{2+} -sensitive current for an average of 7 cells. (C) Current traces of an example cell which does not express I_{K1} in control (black) and with $10\ \mu\text{M}$ BaCl_2 (green). (D) Corresponding current-voltage plot of an average of 3 cells showing no Ba^{2+} -sensitive current.

Single suspended iPSC-CMs have depolarized membrane potentials

While the voltage clamp experiments demonstrated that the cardiac Na^+ and Ca^{2+} channels remain functional after dissociation, recording action potentials from

iPSC-CMs in suspension was challenging. After switching to current clamp mode, the resting membrane potential of iPSC-CMs was typically between 0 and -15 mV. A negative resting membrane potential close to the reversal potential of K⁺ could be achieved by injecting a small constant, negative holding current. After that, action potentials could be triggered with a brief depolarizing stimulus. However, the action potentials were very short and had a monotonic repolarization without a plateau phase, which does not match well with adult human cardiac action potentials (see Figure 4, black trace labeled '-180 pA'). The I_{K1} channels expressed in fully differentiated human cardiomyocytes hyperpolarize the resting membrane potential of these cells, bringing it close to E_{K^+} . An important difference with a constant hyperpolarizing current is that I_{K1} channels close upon strong depolarization, allowing development of the plateau phase of the cardiac action potential. In order to improve our methods, and obtain better action potential recordings from suspended single iPSC-CMs, we implemented the dynamic clamp technique on our automated patch clamp device in order to inject simulated I_{K1} .

Figure 4: Dynamic clamp used to simulate I_{K1} conductance on action potentials (APs) of Cellartis Cardiomyocytes.

The simulated I_{K1} conductance could replace injected current to achieve a native resting membrane potential (RMP) of approximately -94 mV. The cells repolarized faster and the AP duration decreased with increasing I_{K1} conductance. Note: in order to keep a RMP of -94 mV in the absence of simulated I_{K1} conductance, -180 pA of holding current was injected. This was removed upon addition of I_{K1} and RMP remains at -94 mV.

Hybrid models of suspended iPSC-CMs and simulated I_{K1} channels produce longer action potentials with a plateau phase

Our dynamic clamp implementation is in many ways similar to other published implementations^{13, 14, 17}, but differs in two aspects: the functional integration with existing patch clamp control software, and the model used to compute I_{K1} . Earlier work has used I_{K1} formulations that model the current as a steady-state current, *i.e.* without time-dependence. The model by Ishihara *et al.* used in this study is a more detailed, time-dependent model that includes the rectifying effects of Mg^{2+} and polyamines, as well as two modes of channel closure by spermine¹⁵. Including this more detailed model is relevant, as time and voltage dependent Mg^{2+} block or unblock can significantly affect action potential duration¹⁸.

In current clamp experiments with suspended iPSC-CMs, we injected virtual I_{K1} current with varying conductance densities (G_{K1}), depending on the specific cell. At depolarized potentials (> -40 mV), the Ishihara model does not generate much outward current, therefore increasing G_{K1} did not immediately induce hyperpolarization. This could be solved by briefly injecting a hyperpolarizing current, bringing the membrane potential to values inducing a sufficiently large outward I_{K1} current. As a result, the membrane potential was maintained at or close to E_K (which was -95 mV for the simulated I_{K1} channels) due to the injected virtual I_{K1} current. After this, the constant hyperpolarizing current was switched off, as it was only needed to start the experiment.

Action potentials recorded from iPSC-CMs with addition of virtual I_{K1} differed from the earlier recorded brief and monotonically repolarizing action potentials. Injecting I_{K1} resulted in a stable resting membrane potential, fast upstroke velocities and prolonged action potential duration with a clear plateau phase (Figure 4). Further increasing G_{K1} , resulting in injection of more I_{K1} , caused a small additional hyperpolarization, but more significantly, also shortened action potential duration (Figure 5A) and slightly decreased upstroke velocity (Figure 5B). This is consistent with the role of I_{K1} in cardiomyocyte electrophysiology, as it contributes to resting membrane potential stability and the final repolarization phase of the action potential⁴. A benefit of our newly developed approach is that experimental throughput can be moderately increased as we can record from, and inject I_{K1} into, 4 cells in parallel.

Figure 5: Effects of adding simulated I_{K1} on AP parameters.

(A) Adding I_{K1} prolongs the APD_{90} of action potentials recorded from Cellartis Cardiomyocytes ($n=4$) compared to constant current injection. With increasing I_{K1} conductance, the prolongation of the action potential becomes smaller, consistent with the role of I_{K1} in the final repolarization of the cardiac action potential. **(B)** Upstroke velocity of the action potentials after addition of simulated I_{K1} is high, as with constant current injections, and decreases slightly with increasing I_{K1} conductance.

In Figure 6 we show an example of an experiment in which we were able to record APs from 3 iPSC-CMs in parallel (Figure 6A) and have also plotted the injected I_{K1} (Figure 6B). The fourth channel was available, but for this channel no cell was captured successfully. Additional experiments, in which we studied the effect of G_{K1} on upstroke velocity, showed that we could perform a dynamic clamp experiment in 20 out of 28 wells in 7 experimental runs that used 4 wells per run (71% success rate), see Supplementary Figure 3.

Addition of virtual I_{K1} restores sensitivity of the plateau phase to pharmacological modulation

In earlier experiments, it proved difficult to observe effects of Ca^{2+} channel antagonists or agonists on the action potential shape of suspended iPSC-CMs when injecting a

Figure 6: Action potentials recorded simultaneously after adding simulated I_{K1} . (A) Action potentials from 3 cells recorded in parallel on a Patchliner Quattro. (B) Simulated I_{K1} was recorded for each of the 3 cells and is shown. Note the brief increase in I_{K1} during the upstroke of the action potential, where I_{K1} channels respond with increased current to the depolarization induced by the pacing stimulus, which is in line with the slight decrease observed in upstroke velocity with increasing I_{K1} conductance.

of the plateau phase. After observing the restoration of the plateau phase when injecting virtual I_{K1} current, we tested if the action potential became again sensitive to manipulation of the L-type Ca^{2+} current. After injecting I_{K1} (on average G_{K1} was 267 ± 42 pS/pF) we observed an APD_{90} of 118 ± 21 ms ($n=6$), which significantly prolonged after enhancing the L-type Ca^{2+} channel with $1 \mu M$ Bay K 8644 to 155 ± 31 ms ($n=6$, see Figure 7A,B). In contrast, blocking the L-type Ca^{2+} channel with $30 \mu M$ nifedipine caused a shortening to 81 ± 14 ms ($n=6$). These findings demonstrate that enabling the plateau phase of the action potential of suspended iPSC-CMs by addition of virtual I_{K1} channels restores action potential sensitivity to L-type Ca^{2+} channel modulation.

Figure 7: Effects of calcium (ant)agonists on AP duration.

(A) The calcium channel agonist Bay K 8644 increased AP duration while simulated I_{K1} was added (green trace). Conversely, the calcium channel antagonist nifedipine decreased AP duration (gray trace) as compared with control (black trace). In this experiment, I_{K1} was injected at 400 pS/pF and RMP was -94 mV. (B) Average responses from 6 cells showing significantly increased APD₉₀ after exposure to 1 μM Bay K 8644 (* $p < 0.05$) and decreasing APD₉₀ after exposure to 30 μM nifedipine.

Discussion

In this study, as a proof of principle, we have demonstrated that the dynamic clamp technique can be used in combination with automated patch clamp devices, thereby creating a higher throughput alternative to manual patch clamp. Using dynamic clamp to add virtual I_{K1} channels to suspended iPSC-CMs allowed us to record action potentials with waveforms that are more representative of the human fully differentiated ventricular cardiomyocyte. This is especially relevant to the CiPA initiative, which aims to use hSC-CMs in drug safety testing.

Achieving higher throughput evaluation of drug effects on action potentials generated by iPSC-CMs will most likely require the use of isolated, suspended cells, as these can be used with automated patch clamp devices. The depolarized resting membrane

potential of freshly isolated iPSC-CMs is a challenge that can be overcome, at least to some extent, by using the dynamic clamp technique (this study). Dissociation of cardiomyocytes with enzymes disrupting the extracellular matrix, whether native or created in culture, is known to affect the function of I_{Kr} , I_{Ks} and I_{K1} channels^{19, 20}. Hoshino *et al.* demonstrated that the approach used to isolate cardiomyocytes from neonatal mouse hearts has a significant impact on I_{K1} channel function and resting membrane potential. Using enzymatic perfusion of the hearts preserved I_{K1} channels, while the chunk digestion method resulted in four to five times smaller I_{K1} currents and a depolarization of ~ 20 mV. The approach used in this and other studies to obtain single, suspended iPSC-CMs is very similar to the chunk digestion method, and we have indeed observed only small Ba^{2+} -sensitive currents, which may be smaller than those observed in studies using adherent iPSC-CMs^{5, 16, 21}. Further improvement of cell dissociation protocols may improve results with iPSC-CMs on automated patch clamp devices. This is supported by recent work by Rajamohan *et al.* who have used a two-step dissociation protocol and recorded APs from the dissociated iPSC-CMs using both manual and automated patch clamping approaches (same device as used in this study). From the data in this study it appears that the two-step protocol yields cells with a more hyperpolarized membrane potential²².

In the present study, we provide proof-of-principle that automated patch clamp devices and dynamic clamp can be combined successfully. The resulting action potential durations and waveforms are very comparable to those obtained in manual patch clamp experiments in which I_{K1} was added to iPSC-CMs using dynamic clamp^{13, 14}, including the action potential prolongation in response to Bay K 8644. However, more research will be needed to establish the method, and to define its limits and benefits. A better insight into the effects of the specific I_{K1} model that is applied is needed, as well as a well-defined algorithm that allows us to determine which amount of added I_{K1} results in the most predictive results and therefore the best safety pharmacology assay. This should subsequently be demonstrated using the set of drugs defined by CiPA. If these goals can be reached, performing predictive patch clamp experiments with iPSC-CMs with a higher throughput becomes feasible.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

BG: data acquisition, data analysis, data interpretation, revising; NB: data acquisition, data analysis, data interpretation, study design, writing; SS-F, AO: data analysis, data interpretation, revising; TvV, MV, NF: data interpretation, revising; TdB: data acquisition, data analysis, data interpretation, study design, writing, revising. All authors meet the following criteria: 1) substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work, 2) drafting the work or revising it critically for important intellectual content, 3) final approval of the version to be published, 4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary material

Supplementary Figure 1: Dynamic clamp implementation.
Diagram explain our dynamic clamp implementation into the automated patch clamp system using four channels.

Supplementary Figure 2: Flowchart

The flowchart describes the various steps that are taken during the dynamic clamp experiments from preparing the chips to recording action potentials.

Supplementary Figure 3: Effect of G_{K1} on upstroke velocity.

Effect of increasing G_{K1} on upstroke velocity. Dynamic clamp experiments could be performed in 20 out of 28 wells, divided over 7 experimental runs that used 4 wells per run (71% success rate).

Abstract

Human induced stem cell-derived cardiomyocytes (hiPSC-CMs) are a virtually endless source of human cardiomyocytes that may become a great tool for safety pharmacology; however, their electrical phenotype is immature: they show spontaneous action potentials (APs) and an unstable and depolarized resting membrane potential (RMP) because of lack of I_{K1} . Such immaturity hampers their application in assessing drug safety. The electronic overexpression of I_{K1} (e.g. through the dynamic clamp (DC) technique) is an option to overcome this deficit. In this computational study, we aim to estimate how much I_{K1} is needed to bring hiPSC-CMs to a stable and hyperpolarized RMP, and which mathematical description of I_{K1} is most suitable for DC experiments. We compared five mature I_{K1} formulations (Bett, Dhamoon, Ishihara, O'Hara-Rudy and ten Tusscher) with the native one (Paci), evaluating the main properties (outward peak, degree of rectification) and we quantified their effects on AP features (RMP, V_{max} , APD_{50} , APD_{90} (AP duration at 50% and 90% of repolarization) and APD_{50}/APD_{90}) after including them in the hiPSC-CM mathematical model by Paci. Then, we automatically identified the critical conductance for I_{K1} ($G_{K1, critical}$), the minimally required amount of I_{K1} suppressing spontaneous activity. Preconditioning the cell model with depolarizing/hyperpolarizing prepulses allowed to highlight time dependency of the I_{K1} formulations. Simulations showed that inclusion of mature I_{K1} formulations resulted in hyperpolarized RMP and higher V_{max} , and observed $G_{K1, critical}$ and the effect on APD strongly depended on I_{K1} formulation. Finally, the Ishihara I_{K1} led to shorter (-16.3%) and prolonged (+6.5%) APD_{90} in response to hyperpolarizing and depolarizing prepulses, respectively, whereas other models showed negligible effects. Fine-tuning of G_{K1} is an important step in DC experiments. Our computational work proposes a procedure to automatically identify how much I_{K1} current is required to inject to stop the spontaneous activity, and suggests the use of the Ishihara I_{K1} model to perform DC experiments in hiPSC-CMs.

Introduction

To be successfully placed on the market, a drug must be effective (*i.e.* it must be able to hit the desired target at clinical relevant concentrations) and safe (*i.e.* no side effects that could compromise the function of organs should occur). Insufficient efficacy and safety are responsible for almost 60% of the attrition rate in drug discovery and development^{1, 2}. In the cardiovascular area, the proarrhythmic potential of a drug is the side-effect reporting the highest number of post-approval adverse events^{2, 3}. Torsade de Pointes (TdP) is a potentially fatal arrhythmia that may occur when the repolarization of the ventricles is delayed. At the cellular scale, block of the human Ether-à-go-go Related Gene (hERG) channels conducting the rapid delayed rectifier potassium current (I_{Kr}), is among the ones responsible for the delayed repolarization of the ventricles, that is detectable in surface electrocardiogram (ECG) traces as a prolonged QT interval.

The International Committee on Harmonization S7B (ICH S7b, preclinical) and the E14 (clinical) guidelines successfully reduced the risk of approving drugs that have the potential to induce TdP, by deprioritizing development of drugs that block hERG channels or cause QT prolongation. However, the guidelines are affected by low specificity⁴. To overcome this problem, the Comprehensive *in vitro* Proarrhythmic Assay (CiPA) initiative suggests an alternative paradigm to assess the safety of a new compound in the preclinical and early clinical stages^{4, 5}. The paradigm shift consists of the hybrid combination of *in vitro* and *in silico* human specific models⁵. The first step in the CiPA paradigm focuses on the evaluation of the functional drug effects on inward currents involved in the plateau phase, such as the long lasting calcium current (I_{CaL}) and the late sodium current (I_{NaL}), and outward currents active during repolarization, such as the slow delayed rectifier potassium current (I_{Ks}), the inward rectifier potassium current (I_{K1}) and the transient outward potassium current (I_{to}). At the second stage, the experimental data are included into an *in silico* human adult ventricular action potential (AP) model⁶ that allows one to investigate the behavior of the affected currents in an integrated environment. Finally, the prediction of the drug's effects is confirmed or disproved through *in vitro* experiments using human induced pluripotent stem cells (hiPSC) derived cardiomyocytes (hiPSC-CMs). Furthermore, *in silico* hiPSC-CM models have been successfully employed to assess drug efficacy. Paci *et al.*⁷ showed that mexiletine and ranolazine, two multichannel blockers that target I_{Na} , I_{NaL} , I_{CaL} and I_{Kr} are able to recover the AP duration (APD) to physiological values in a LQTS3 hiPSC-CM population. *In silico* models of hiPSC-CMs also help to quantitatively characterize the differences between hiPSC-CMs and adult ventricular

cardiomyocytes⁷. Indeed, the simulation of the pharmacological block of ion currents showed that the two cells types respond in different ways⁸, giving a quantitative description of such a difference.

hiPSC-CMs are a promising tool for drug efficacy and safety testing. Because hiPSCs are obtained by reprogramming adult somatic cells to a pluripotent state, they are a virtually limitless source of cells with the same genome as the donor. Their origin from human adult cells avoids ethical issues associated with the use of human embryonic stem cells, bridge the gap resulting from the use of animal models and bring personalized medicine closer. However, hiPSC-CMs are characterized by an immature electrophysiological phenotype that limits their employment in drug safety assessment⁹. Unlike adult ventricular cardiomyocytes, they show high levels of expression of the HCN gene family (that encodes for the pacemaker current I_p), and low levels of the KCNJ2 gene, responsible for I_{K1} . The balance between ion channel types expressed by iPSC-CMs results in a depolarized resting membrane potential (RMP), a slow upstroke velocity (V_{max}) due to the inactivation of sodium channels, and the absence of the plateau phase. Furthermore, due to the contribution of I_p , hiPSC-CMs show spontaneous automaticity⁹.

Several studies¹⁰⁻¹² have demonstrated that compensating for the low levels of I_{K1} improves the electrophysiological phenotype of hiPSC-CMs, making its phenotype more mature and more relevant for drug safety testing. Apart from attempts to improve differentiation protocols, two direct methods have been used to increase functional expression of I_{K1} in hiPSC-CMs (*e.g.* through adenoviral overexpression of I_{K1} channels¹³), or by applying the dynamic clamp (DC) technique to insert virtual I_{K1} conductance^{14, 15}. DC is a refinement of the traditional patch clamp. It allows to interface the membrane potential of (one or more) cells with a computer running a real-time simulation of ion channels or gap junctions. In this way, it's possible to create a virtual electrical connection between cells, or add a virtual ion channel to the cell membrane through a computational model that describes the time course of that current in response to membrane potential of the connected cell(s). The injected current can be fully described by mathematical equations and parameters. Through the tuning of the parameters, the amount of injected current can be modulated precisely, in order to adapt it to the cell under investigation. The ability to fine-tune ion channel conductance to each cell being tested is essential when adding I_{K1} to hiPSC-CMs, because too little will leave the cells beating spontaneously, whereas too much I_{K1} can render the cell non-excitable. Earlier work by Verkerk *et al.*¹¹ indeed reported that the injection of I_{K1} eliminates the spontaneous activity of hiPSC-CMs and

provides a more physiological phenotype¹¹. Bett *et al.*¹² highlighted that hiPSC-CMs respond like adult ventricular CMs to the administration of Bay K 8644 only if I_{K1} is injected¹². Studies testing DC strategies to mature hiPSC-CMs have almost exclusively used manual patch clamping, but recently our group successfully implemented DC-based I_{K1} injection in hiPSC-CMs on an automatic patch clamping platform that is capable of medium to high-throughput drug screening by recording from up to 8 cells in parallel^{16, 17}.

Fine-tuning of the I_{K1} conductance (G_{K1}) is an important step in DC experiments, whether manual or automated patch clamping is used. Higher throughput applications however require an automated tuning procedure that can run unattended. In this study we propose an approach to establish the required $G_{K1, \text{critical}}$ in a way that can be implemented on automated patch clamping platforms. We define $G_{K1, \text{critical}}$ as the minimal value of conductance that is sufficient to bring the cell to a stable and hyperpolarized RMP. Identifying $G_{K1, \text{critical}}$ is relevant because it allows to estimate the minimum amount of the injected I_{K1} for a cell, and it can subsequently be used as a reference value when G_{K1} is up- or downscaled, facilitating comparison of results obtained from different cells. Next to the value of G_{K1} , the time- and voltage-dependent properties of the used computational model of I_{K1} affect the AP waveform. Several studies have captured the electrophysiology of I_{K1} in a computational model, providing descriptions with different levels of detail^{6, 12, 18-21}. The choice of the I_{K1} model used in DC experiments may therefore affect the resulting action potentials.

In this study, we have employed *in silico* DC experiments to explore the impact of I_{K1} on the immature hiPSC-CM electrophysiologic phenotype. Our simulations have the aim to 1) compare the effects on AP waveform of the six different I_{K1} formulations^{6, 12, 18-21} in the hiPSC-CM computational model developed by Paci *et al.*²², 2) establish an algorithm to estimate $G_{K1, \text{critical}}$ for the six models, 3) assess how the up- and downscaling of G_{K1} relative to $G_{K1, \text{critical}}$ affects the AP waveform, and 4) study the effects of hyper- or depolarizing prepulses on AP waveform.

Methods

hiPSC-CM model, I_{K1} formulations and identification of $G_{K1, \text{critical}}$

We carried out the *in silico* experiments using the computational model of the hiPSC-CM developed by Paci *et al.*²² The Paci model is robustly constrained by experimental data obtained by Ma *et al.*²⁰, who electrophysiologically characterized hiPSC-CMs. Furthermore, they quantitatively investigate the mechanisms responsible for the

immature electrophysiological phenotype, assessing the effects on spontaneous activity and AP waveform through the replacement of a subset of native currents (I_{Na} , I_{to} , $I_{Ca,L}$, I_{K1} , I_{Kr} , I_{Ks}) with adult ventricular current formulations from the O'Hara-Rudy model⁶. The aforementioned characteristics make it a suitable model for evaluating DC strategies through adding I_{K1} conductances to hiPSC-CMs. In this study, we have evaluated all I_{K1} model structures available in the literature, namely, those published by Bett *et al.*¹², Dhamoon *et al.*¹⁸, Ishihara *et al.*¹⁹, Paci *et al.*²², O'Hara *et al.*⁶ and ten Tusscher *et al.*²¹. The six I_{K1} formulations mainly differ in the presence (or absence) of time dependency in their kinetics and how the rectification of the outward current is described. A more detailed comparison between the six I_{K1} formulations is reported in Table 1 and Figure 1. We set the extracellular potassium concentration $[K^+]_o$ to 5.4 mM, and the intracellular concentration $[K^+]_i$ to 150 mM, setting the reversal potential for the six currents at $E_K = -88.8$ mV. In order to study how the different formulations of I_{K1} affect the AP waveform, we paced hiPSC-CM cell models at 1 Hz, with a current stimulus of amplitude 15.2 pA/pF and 5 ms of duration, able to elicit APs in all the six models under comparison. The AP waveforms were quantitatively described through five biomarkers: the RMP, the maximal speed of depolarization during the upstroke (V_{max}), the AP duration at 50% and 90% of repolarization (APD_{50} and APD_{90}), and the ratio APD_{50}/APD_{90} , a biomarker to describe triangulation.

Figure 1: Comparison of the I_{K1} I-V curves of the six I_{K1} models for the original (published) G_{K1} value.

E_K is marked by the vertical dashed line.

Table 1: Main characteristics of the six I_{K1} formulations

I_{K1} formulation	Experimental data source	Transient	Steady-state	Rectification	Conductance	Reference
Bett	Adult human ventricular cells	NP	Steady-state only	Voltage-dependent	Constant	12, 36
Dhamoon	Guinea pig Kir2.1 in HEK293	NP	Steady-state only	Voltage-dependent	Constant	10, 18
Ishihara	Mouse Kir2.1 in HEK293	V	V	Two channel populations sensitive to $[Mg^{2+}]_i$ and $[SPM]_i$	$[K^+]_o$ dependent	19, 37
O'Hara-Rudy	Adult human ventricular cells	Inactivation gate	V	Voltage-dependent & $[K^+]_o$ dependent	$[K^+]_o$ dependent	6
Paci	Human iPSC-CM	NP	Steady-state only	Voltage-dependent	$[K^+]_o$ dependent $G_{K1} \times 1.1$	20, 22
ten Tusscher	Adult human ventricular and atrial	NP	Steady-state only	Voltage-dependent	$[K^+]_o$ dependent	21, 36

NP: not present, v: implemented

The identification of $G_{K1, \text{critical}}$ for each I_{K1} formulation was performed using the bisection algorithm in the unpaced hiPSC-CM. We performed the search in a range from 0 to 10 times the original (published) value. If the membrane potential of the cell was < -40 mV and with a difference between the minimum and maximum values over a period of 50 seconds that was < 1 mV, the cell was defined as quiescent and the current G_{K1} was stored as $G_{K1, \text{high}}$. On the contrary, if the cell showed spontaneous activity or failed to repolarize, G_{K1} was stored as $G_{K1, \text{low}}$; G_{K1} employed in the next step was calculated as $(G_{K1, \text{high}} + G_{K1, \text{low}})/2$. The G_{K1} value that made the cell quiescent and hyperpolarized was then challenged with a single external stimulus and the bisection search was carried out again. The algorithm stopped when the difference between $G_{K1, \text{high}}$ and $G_{K1, \text{low}}$ was lower than a tolerance set to 0.1% of the original value of G_{K1} .

As a proof of principle, *in vitro* experiments were carried out to determine $G_{K1, \text{critical}}$ in hiPSC-CMs in a dynamic clamp experiment using a custom dynamic clamp system as described in Goversen *et al.*¹⁶ Manual patch clamp recordings were done at 37°C and followed the protocol outlined in Figure S5. Coverslips with hiPSC-CMs were superfused with a bath solution containing (in mM), NaCl 130, KCl 4, CaCl₂ 1.8, MgCl₂ 1.2, NaHCO₃ 18, HEPES 10, Glucose 10. Pipettes had resistances between 2-4 MΩ

when filled with a pipette solution containing (in mM) KCl 10, K-Gluconate 125, CaCl_2 0.6, MgCl_2 2, HEPES 5, Sucrose 30, Na_2ATP 4, EGTA 5. Liquid junction potential (+13.8 mV) was calculated using pClamp 10 and corrected *a priori*. The effect of increasing or decreasing G_{K1} was investigated by running AP simulations paced at 1 Hz, using values for G_{K1} in a range from 0 to 10-fold $G_{K1, \text{critical}}$, increasing G_{K1} of $0.25 \times G_{K1, \text{critical}}$. The last protocol aimed to reproduce the experiment by Ishihara *et al.*²³, using hyper/depolarizing current prepulses. Before the pacing stimulus that triggered the AP, 50 ms current prepulses of 5 pA/pF and -0.5 pA/pF were applied in order to respectively hyperpolarize or depolarize the voltage membrane. The combination of the prepulses and the pacing stimulus was administered at 1 Hz for 30 s.

Comparing the influence of cell-to-cell-variation between iPSC-CMs and between I_{K1} formulations

The Paci 2013 model is based on the data collected by Ma *et al.*²⁰ using hiPSC-CMs. To address the variability between cells that is encountered in experiments, we identified $G_{K1, \text{critical}}$ for each I_{K1} formulation under investigation in a population of 22 cell specific iPSC-CM models, published earlier by Lei *et al.*²⁴ In brief, Lei *et al.* adapted the Paci 2013 model on the base of voltage clamp experiments carried out on hiPSC-CMs, by scaling the maximal conductance (S/F) of I_{Na} (x0.69), I_{CaL} (x0.80), I_{Ks} and the maximal activity of the I_{NaCa} (pA/pF) (tailored for each cell, the values are reported in Table 2). Next, we assessed the variability of the AP morphology while pacing the cell-specific models at 1 Hz.

Hardware and software

The Paci-based hiPSC-CM models including the six I_{K1} formulations were encoded in CellML and solved using OpenCOR (v0.6)²⁵, and on the Cardiac Electrophysiology Web Lab (available at <https://travis.cs.ox.ac.uk/FunctionalCuration/>)²⁶. Simulations done using OpenCOR were performed on a macOS High Sierra (10.13.6) Apple computer (Apple, Cupertino, CA, USA) equipped with a 2.9 GHz quad-core Intel Core i7 (intel, Santa Clara, CA, USA). We used a variable step method for stiff problems (Back differentiation formula) implemented in the CVODE library. The currents and the state variables of the models were stored and displayed with a 0.1 ms time step. The cell models achieved steady-state conditions when the difference of the norm of the state variable vector at the beginning and at the end of the AP was lower than 10^{-6} . The extraction of AP waveform features and plots were performed in Matlab (release R2018a, The Mathworks Inc., Natick, Ma, USA).

Table 2: AP waveform parameters of hiPSC-CM models including the six I_{K1} formulations at $G_{K1, \text{original}}$

Model	RMP (mV)	V_{max} (V/s)	APD ₅₀ (ms)	APD ₉₀ (ms)	APD ₅₀ /APD ₉₀
Paci (unpaced)	-76.9	24.9	366	487	0.75
Bett	-86.8	157.5	58	153	0.38
Dhamoon	-88.1	160.9	101	133	0.76
Ishihara	-87.8	157.9	84	163	0.57
O'Hara-Rudy	-20.4	-	-	-	-
Paci	-77.2	57.9	302	453	0.67
ten Tusscher	-86.1	136.4	258	431	0.60

RMP: resting membrane potential, V_{max} : maximal upstroke velocity, APD_x: action potential duration at x% of repolarization, APD₅₀/APD₉₀: APD ratio, provides a quantification of the shape of the AP waveform.

Results

hiPSC-CM AP waveform is strongly influenced by I_{K1} model structure

A first comparison between the six I_{K1} formulations was made by plotting the steady-state current generated by the models at voltages between -120 mV and +10 mV (see Figure 1). The Dhamoon model shows the highest outward peak (8.5 pA/pF at -69.5 mV), whereas I_{K1} from O'Hara-Rudy shows the lowest outward peak current density (0.91 pA/pF at -77.1 mV). The Paci model, constructed using experimental data obtained from immature iPSC-CMs, is remarkably different from the other models that are based on experimental data from adult myocytes or heterologous expression systems, and reaches the outward peak (0.99 pA/pF) at more depolarized potentials (-46.1 mV). The model structures also differ in the rectification: the Dhamoon model shows I_{K1} density close to zero at depolarized potentials (membrane potential (V_m) > 0 mV), whereas the O'Hara-Rudy model already reaches small current densities around -40 mV. The Bett model differs markedly at positive potentials, showing a clear positive linear growth.

Next, we simulated paced APs using the Paci models incorporating the six I_{K1} models. Analysis of AP waveform allows quantification of the effects of the I_{K1} formulations. The cell models that include the Dhamoon and the Paci I_{K1} formulations show the most hyperpolarized and depolarized RMPs, and the fastest and the slowest V_{max} (-88.1 mV, 160.9 V/s, and -77.2 mV, 57.9 V/s, respectively). The key role played by I_{K1} in repolarization becomes clear when comparing APD₅₀ and APD₉₀ values. On one

hand, the activity of I_{K1} during the plateau and phase 3 of the Bett model leads to shorter action potential durations ($APD_{50}=58$ ms, $APD_{90}=153$ ms). On the other hand, in the model including the O'Hara-Rudy formulation, the low amplitude of I_{K1} is not sufficient to successfully repolarize the membrane potential that settles to a stable but depolarized RMP (-20.4 mV), as also reported by Paci *et al.*²²

Calculating the ratio of APD_{50} and APD_{90} (APD_{50}/APD_{90}) gives some additional insight about the repolarization course. Values close to 0.5 result from APs with a triangular shape for which the plateau phase is virtually absent and the repolarization is monotonic, whereas values close to 1 occur when phase 3 is steep, and thus the final phase of repolarization is fast. The Bett model shows a value ($APD_{50}/APD_{90}=0.38$) that is coherent with a triangular waveform, whereas the Dhamoon model has an $APD_{50}/APD_{90}=0.76$, consistent with a steep phase 3 repolarization. The Ishihara, the Paci and the ten Tusscher models show $APD_{50}/APD_{90} > 0.5$ (0.57, 0.67, 0.60 respectively).

To get a better understanding of the influence of the six models on emerging AP waveforms, we compared the AP waveforms and underlying I_{K1} current profiles (see Figure 2). This showed a clear effect of I_{K1} model structure on AP waveform. The Dhamoon I_{K1} shows the largest outward peak (8.5 pA/pF), as expected from the steady-state I-V characteristic. The Bett current is active throughout all AP phases, but mostly during the plateau phase (~ 1 pA/pF), during which the other models produce currents of negligible amplitude. The Ishihara I_{K1} contributes to the early phase 3 of the AP, starting earlier during the repolarization phase than the Dhamoon, Paci and ten Tusscher I_{K1} models. The Dhamoon model shows the largest peak (8.5 pA/pF) during late repolarization, where it contributes to a fast final repolarization towards the RMP. The Dhamoon and the Ishihara models both bring the RMP (-88.1 and -87.8 mV respectively) close to E_K (-88.8 mV) by conducting a current density of ~ 0.8 pA/pF. The lowest I_{K1} density during phase 4 is generated by the Paci model, which also shows the least hyperpolarized RMP (-77.2 mV).

Scaling relative to $G_{K1, critical}$ facilitates comparison of I_{K1} models or cells

The critical value of G_{K1} ($G_{K1, critical}$) is an estimation of the minimal number of I_{K1} channels that is required on the cell membrane to suppress automaticity, to make the cell 1) quiescent, 2) with an RMP that is stable and close to E_K , even when the cell is perturbed with an external stimulus. After establishing $G_{K1, critical}$ for all six I_{K1} models, we observed two different behaviors.

Figure 2: AP waveforms.

(A) Action potential waveforms elicited by pacing the Paci hiPSC-CM model incorporating the six different I_{K1} at 1 Hz, with the original G_{K1} . The bottom trace displays the original hiPSC-CM Paci model without pacing, which shows the spontaneous activity that is typical of hiPSC-CMs. (B-G) V_m and I_{K1} time courses of the hiPSC-CM cell models including the six I_{K1} formulations. The model including O'Hara-Rudy I_{K1} formulation failed to repolarize after 70 s; here we report the transition from successful to failing repolarization (4th trace in A and E).

When incorporating the Bett, Dhamoon, Ishihara or ten Tusscher models, the hiPSC-CM models become quiescent with G_{K1} values that are lower than those in the original I_{K1} models (see Table 3) with a decrease of 31.4, 87, 60.4 and 41.3% respectively. On the other hand, the Paci and O'Hara-Rudy models needed an increase of G_{K1} (+53.8 and +46.6% respectively) in order to stabilize the RMP; this means that these two models do not provide enough I_{K1} in their original formulation to stop the automaticity. The time course of membrane potential and I_{K1} are depicted in Supplementary Figure S2.

Figure 3 (A-F) depicts the AP waveform with G_{K1} values ranging between 0 and $10 \times G_{K1, \text{critical}}$. The downscaling of G_{K1} resulted in longer APDs and a depolarized RMP when the cells were able to repolarize. Progressively increasing G_{K1} resulted in a faster repolarization process with a smaller APD_{50} and APD_{90} (Figure 3G, H). A higher amount of I_{K1} is also responsible for a hyperpolarized and stable RMP (Figure 3I) that gets close to E_K . The inset in Figure 3I shows that for a conductance corresponding to $G_{K1, \text{critical}}$ RMPs are still quite different: the hiPSC-CM model including the Paci formulation is the most depolarized (-80.1 mV), whereas the model including the O'Hara-Rudy formulation is the most hyperpolarized (-86.7 mV). For all I_{K1} models, the upstroke velocity V_{max} (Figure 3J) increased with increasing G_{K1} valued from ~ 0.5 to $2 \times G_{K1, \text{critical}}$ and then stabilizes at values around 150 V/s for larger G_{K1} values. For $G_{K1} > 7.5 \times G_{K1, \text{critical}}$ and $> 9.25 \times G_{K1, \text{critical}}$ respectively, the pacing stimulus was no longer sufficient to trigger an AP when using the Ishihara or the O'Hara-Rudy models (see insets C and D). Together with $G_{K1, \text{critical}}$, this behavior suggests that there is a range for the amount of I_{K1} that should be injected into the cell, providing indications for *in vitro* experiments.

$G_{K1, \text{critical}}$ shows limited variability between cell-specific iPSC-CM models

Variation in AP waveform between hiPSC-CMs is well-known, and this may influence the $G_{K1, \text{critical}}$ value required in a DC experiment for maintaining quiescence of a particular cell. To test this, we employed 22 cell-specific iPSC-CM models, tailored on the Paci 2013 model, with variations in ion currents based on voltage clamp data²⁴. For each cell-specific model, we identified $G_{K1, \text{critical}}$ using the six I_{K1} formulations we wanted to assess. Figure 4 illustrates the range of the 22 $G_{K1, \text{critical}}$ values obtained for each I_{K1} formulation. $G_{K1, \text{critical}}$ did not follow a normal distribution, therefore we described them through median, 25th and 75th percentile. Median $G_{K1, \text{critical}}$ was 598.6 (I_{K1} according to Bett), 104.2 (Dhamoon), 827.2 (Ishihara), 28.2 (Paci), 251.2 (O'Hara-Rudy) and 2510 S/F (ten Tusscher).

Figure 3 (A-F): AP waveforms at varying G_{K1} .

Note how a G_{K1} value that is too large prevents the pacing stimulus from triggering an AP for cell models that include Ishihara¹⁹ and O'Hara-Rudy *et al.*⁶ I_{K1} (insets in C and D). Light colors code for low G_{K1} values, dark colors for high G_{K1} values.

(G-J): AP features. AP features (APD₅₀, APD₉₀, RMP and V_{max}) extracted from the membrane potential time courses. Simulations were run using G_{K1} values ranging from 0 (no I_{K1}) to $10 \times G_{K1, \text{critical}}$ and increase with $0.25 \times G_{K1, \text{critical}}$ step. The experimental range for APD₅₀ and APD₉₀ refers to adult healthy cardiomyocytes from Britton *et al.*³⁰

Table 3: AP features of the 1 Hz paced hiPSC-CM models for $G_{K1} = G_{K1, \text{critical}}$

Model	$tt_{K1, \text{original}}$ (S/F)	$tt_{K1, \text{critical}}$ (S/F)	Dtt_{K1} (%)	RMP (mV)	V_{max} (V/s)	APD_{50} (ms)	APD_{90} (ms)	APD_{50}/APD_{90}
Bett	1000	685.8	-31.4	-85.9	145.0	85	242	0.35
Dhamoon	1000	129.4	-87	-84.0	112.8	301	541	0.56
Ishihara	2500	989.7	-60.4	-85.9	132.3	238	458	0.52
O'Hara-Rudy	190.8	279.8	+46.6	-86.7	112.8	349	686	0.51
Paci	28.149	43.3	+53.8	-80.1	85.1	242	367	0.66
ten Tusscher	5405	3170	-41.3	-83.7	108.2	306	546	0.56

$tt_{K1, \text{original}}$: default value of Kir2.1 conductance, $tt_{K1, \text{critical}}$: critical value of Kir2.1 conductance, that brings RMP to stable and hyperpolarized values, Dtt_{K1} : variation (in %) between original and critical value of tt_{K1} .

Within the six populations of cell-specific models we found a limited range for $G_{K1, \text{critical}}$: the population including the I_{K1} according to Paci reported the largest variation of the 25th and the 75th percentile from the median $G_{K1, \text{critical}}$ (-2.47% and +2.82%, respectively), whereas the models including the other I_{K1} formulations showed variations lower than 1%. Table S1 reports in detail the cell-specific $G_{K1, \text{critical}}$ identified for each cell.

To test if our findings with the cell-specific models were reflected in *in vitro* experiments, a small set of dynamic clamp was done using hiPSC-CMs. We injected I_{K1} using the formulation by Ishihara, and determined $G_{K1, \text{critical}}$ (see Figures S5 and S6). In *in silico* experiments conditions are ideal, and we were able to check if the membrane potential was stable and in steady-state during a 50 s long time window, for many tested G_{K1} values. During *in vitro* experiments, membrane potential is less stable because of noise, and testing many 50s iterations of the bisection algorithm takes more time than typically feasible in patch clamp experiments. For the aforementioned reasons, we did not implement a bisection algorithm, but instead started from a large G_{K1} value (5000 S/F) which was decreased in steps of 500 S/F. The average $G_{K1, \text{critical}}$ was 2750 ± 660 S/F (n=4), with values ranging from 1000 to 4000 S/F, confirming the cell-specific nature of the $G_{K1, \text{critical}}$ parameter. In three additional cells, slightly more than 5000 S/F was needed to suppress minimally remaining automaticity, but the correct value could not be obtained before the experiment expired.

The comparison between the median $G_{K1, \text{critical}}$ found in the cell-specific models and the one identified in the initial models used earlier (Figure 4B) helps to get more insight in the differences between cells.

Figure 4: Critical G_{K1} varies more between formulations than between cells.

In the cell-specific models, for all the included I_{K1} formulations, the $G_{K1, \text{critical}}$ value observed was lower than the one identified in the initial Paci 2013 models. The distance between the initial Paci 2013 and the cell-specific models was the highest in the models including I_{K1} according to Paci (-38.2%), whereas it reached -12.3% when the models included the O'Hara-Rudy I_{K1} formulation. The difference among $G_{K1, \text{critical}}$ in the other I_{K1} formulations was within the boundaries set by Paci and O'Hara-Rudy I_{K1} models.

We further assessed the behavior of the cell-specific models by eliciting APs with external stimuli at 1 Hz, using the cell-specific $G_{K1, \text{critical}}$. RMP was hyperpolarized, close to E_K , and within a limited range (Figure 5A and Figure S3), consistent with the low variability of $G_{K1, \text{critical}}$. I_{K1} contributes to stabilization of the RMP. We compared the contribution of the I_{K1} formulations by measuring the average I_{K1} current density during the diastolic interval, just before the external stimulus (Figure 5B). The amount of current is comparable between the six I_{K1} formulations, but consistent with the less negative RMP, the population that includes the Paci I_{K1} model reported a lower average I_{K1} density during the diastolic interval. APD₅₀ and APD₉₀ showed stronger variability (Figure 5C, D), which is consistent with the cell-specific variations in I_{Ks} and I_{NaCa} current densities. The population including I_{K1} according to Bett showed the shortest APD₉₀ (287 ms), whereas the others are included within 300 and 450 ms. For a detailed overview of the AP waveform of the cell-specific models, see Figure S3 and Table S1.

Figure 5: Main AP features of the cell specific models paced at 1 Hz.

(A) RMP are within a limited range. The population of models including I_{K1} according to Paci showed a less negative RMP ($RMP_{median} = -77.4$ mV). (B) Average I_{K1} density during the last 50 ms of the diastolic interval, before the stimulus; due to the less negative RMP, the current density is lower in the population that includes the Paci I_{K1} . (C-D) APD_{50} and APD_{90} show a higher variability within the populations.

Reproducing the effect of prepulses on APD requires time dependence of I_{K1}

While most I_{K1} models do not include a time constant in the equations describing rectification, experimental data has demonstrated an effect of time-dependence of I_{K1} channel rectification²³, as brief prepulses given before the pacing stimulus have been shown to affect AP duration in guinea pig ventricular cardiomyocytes. Specifically, a hyperpolarizing prepulse caused faster repolarization, *i.e.* a shorter APD, whereas a depolarizing prepulse led to APD prolongation. It was demonstrated that I_{K1} was the current underlying this behavior because of the different availability of open channels at hyperpolarized/depolarized membrane potentials. We tested whether prepulses affect the APD in the initial hiPSC-CM models with the six different I_{K1} models, because the contribution of I_{K1} to variation in repolarization duration can affect the outcome of DC experiments aimed at testing proarrhythmic properties of drugs. Figure 6A1-6 and Figure 6B1-6 depict the AP waveforms and I_{K1} profiles of the last beat of a train of paces of the six hiPSC-CM models. At first sight, it is clear that the hiPSC-CM model including the Ishihara I_{K1} formulation is the only one to show significant effects on AP, as the hyperpolarizing current prepulse shortened APD_{90} (-16.3%), whereas the depolarizing prepulse caused APD_{90} prolongation (+6.5%) with respect to the AP elicited with no prestep, reproducing qualitatively the experimental

data. The effects observed in the other AP models were negligible (see Table S2). For the O'Hara-Rudy model this was surprising, as this I_{K1} model does include a time constant in the description of rectification by including an instantaneous rectification factor (R_{K1}, ∞) and an inactivation gating variable (x_{K1}, ∞). In the simulations of hiPSC-CMs with the O'Hara-Rudy I_{K1} , closer inspection of the behavior of these parameters showed that x_{K1} was only minimally affected by the prepulses, ranging between 1 and 0.993 (minimum value obtained with the hyperpolarizing prepulse, see Figure S4G-I). Figure 6C1-6 shows I_{K1} phase plots, in which I_{K1} density is plotted against V_m during repolarization, similar to a phase diagram depicting I_m vs. V_m . The Ishihara formulation provided different amounts of I_{K1} in response to the prepulses. In dynamic conditions, the I_{K1} phase plots are different from I_{K1} in steady-state, showing a larger amount of I_{K1} available also at more depolarized potentials. In particular, when the cell is preconditioned with a hyperpolarizing prepulse, it is possible to notice a further smaller I_{K1} peak (0.66 pA/pF) at -38.5 mV responsible for the APD shortening. In contrast, no appreciable differences were observed with the other models in which the I_{K1} phase plots overlapped the steady-state I-V curve. Therefore, the I_{K1} formulation that we consider the most suitable to implement in DC experiments is the one according to Ishihara *et al.*¹⁹.

Discussion

hiPSC-CMs are a promising tool for drug safety screening and to study cardiac arrhythmia mechanisms. They represent a pillar in the CiPA initiative and allow to study the effects of drugs on AP morphology in an integrated cellular environment with similarities to human adult cardiomyocytes. the main characteristics that hampers their application in assessing drug safety is their spontaneous activity that leads to an unstable and depolarized RMP ($V_m > -60$ mV). Previous studies demonstrated that it is possible to improve the 'immature' phenotype of hiPSC-CMs through the overexpression of I_{K1} either by virally overexpressing I_{K1} channels in the cells¹³, or through 'electronic expression' using the DC technique^{10-12, 16}. DC is a powerful technique because it is able to mimic I_{K1} carried by biological ion channels. Although it is not able to reproduce the molecular interactions that real I_{K1} channels can participate in, DC does allow fine control of the amount of I_{K1} added and allows the experimentalists to change it 'on the fly', adapting it to characteristics (e.g. maturity, AP waveform, cell size) of the cell under investigation.

Figure 6: A1-6, B1-6: Membrane potential and I_{K1} waveforms for the hiPSC-CM models including the six I_{K1} formulations during the last beat. The cell models were paced at 1 Hz for 30 s. The hyperpolarizing and the depolarizing current prepulse had an amplitude of 5 pA/pF and -0.5 pA/pF respectively and were applied for 50 ms before the AP triggering pulse. Note that only the model including the Ishihara I_{K1} formulation shows significant effects on AP duration. (C1-6) I_{K1} phase plots of I_{K1} plotted against membrane potential during repolarization (blue, red and yellow solid lines) and in steady state (black dashed lines). Arrows point from the start of repolarization towards the end. The Ishihara I_{K1} formulation provides outward current at more depolarized potentials during the repolarization phase. Such outward current contribution is not present in steady state conditions, where I_{K1} density is much smaller at depolarized potentials. No differences between dynamic I-V curves during repolarization and during voltage clamp protocol (steady state conditions) were observed in the other models.

Our study aims to answer two main questions: 1) how much I_{K1} has to be injected to stop the automaticity with a stable RMP? and 2) which is the most suitable I_{K1} mathematical formulation to employ during *in vitro* DC experiments? We addressed these two questions through a computational approach, using the Paci *et al.* model²² as a virtual hiPSC-CM and simulating DC experiments. To this end, we 1) compared a subset of I_{K1} formulations present in literature and we assessed how they affect the AP waveform of the simulated hiPSC-CM, 2) identified the minimal amount of virtual I_{K1} channels needed to stop the automaticity through the parameter $G_{K1, critical}$, 3) investigated the effects on AP waveform features of down/upscaling of I_{K1} , and 4) tested the capability of the I_{K1} formulations to reproduce experimental data reporting APD dependency on hyper- or depolarizing prepulses through an I_{K1} specific mechanism.

I_{K1} formulation and its effects on AP waveform

The comparison of the steady-state I-V curves highlights the differences between the I_{K1} formulations under investigation. The original Paci *et al.*²² model includes I_{K1} , characterized by a low outward peak current at depolarized potentials (~ -46 mV). The other I_{K1} models derived from adult cardiomyocyte models show substantial differences in the outward peak current or in the degree of rectification. The peak outward current density ranges between 1 (O'Hara-Rudy *et al.*⁶) and ~ 8 pA/pF (Dhamoon *et al.*¹⁸). The I_{K1} formulation according to Bett *et al.*¹² shows a low degree of rectification, with substantial I_{K1} density also at more depolarized potentials. The Ishihara *et al.*¹⁹ I_{K1} formulation has a more complex mathematical formulation compared to the other models, and quantitatively describes how the block exerted by intracellular cations as Mg^{2+} and polyamines can affect the ion channel gating. *In silico* experiments allowed us to assess the effects of the aforementioned properties on the AP waveform, avoiding disturbances due to the high variability of AP waveform in real hiPSC-CMs¹¹. When the evaluated I_{K1} formulations replace the native one in the Paci *et al.*²² model, RMP becomes stable and hyperpolarized. This holds true for models with all tested I_{K1} formulations when paced at 1 Hz, but the I_{K1} formulation according to O'Hara-Rudy *et al.*⁶ leads to a stable but depolarized RMP (~ -20.4 mV), as shown by Paci *et al.*²² These results highlight that the amount of I_{K1} provided by the O'Hara-Rudy *et al.*⁶ in the original formulation is not enough to stabilize RMP to hyperpolarized values. Simulations confirmed the link between hyperpolarized RMP and V_{max} ; the overexpression brings the cell to a different working point (*i.e.* the gating variables are in a different state), unveiling I_{Na} that is responsible for a faster upstroke. A strong I_{K1} is responsible for a faster repolarization (see the steep phase 3 in the hiPSC-CM model including the formulation according to Dhamoon *et al.*¹⁸), and this characteristic, together with a strong rectification, leads to APD_{50}/APD_{90} ratios > 0.5 . On the other hand, the low degree of rectification in the Bett *et al.*¹² formulation is responsible for a substantial current (~ 1 pA/pF) at depolarized potentials that abolished the plateau phase. Our results are in agreement with the study by Verkerk *et al.*¹¹, that included in the Paci *et al.* model the I_{K1} formulations according to Meijer van Putten *et al.*¹⁰, Bett *et al.*¹² and Rocchetti *et al.*²⁷ In their simulations they reported a stable and hyperpolarized RMP, a faster V_{max} and a faster repolarization. As reported in our simulation, they also showed that Bett *et al.* I_{K1} dramatically shortened the APD.

How much I_{K1} should be injected to suppress spontaneous activity?

Earlier studies employing DC to inject I_{K1} in hiPSC-CMs have typically standardized the amount of added I_{K1} relative to cell capacitance (*e.g.* a fixed peak outward current density). In this study, we have described an alternative approach based on the critical

G_{K1} , defined as the parameter that depicts the minimal number of I_{K1} channels on the cell membrane needed to bring the membrane potential to hyperpolarized and stable values, thereby suppressing the spontaneous activity. Our DC experiments in hiPSC-CMs indeed confirm that different cells have a different $G_{K1, critical}$ value (Figure S6).

In our simulations, the identification of $G_{K1, critical}$ for each model showed a substantial decrease (up to -87%) for the I_{K1} formulations according to Bett *et al.*¹², Dhamoon *et al.*¹⁸, Ishihara *et al.*¹⁹, and ten Tusscher *et al.*²¹. AP waveform analysis of the simulated AP highlights the correlation between G_{K1} and RMP: a higher G_{K1} leads to a more hyperpolarized RMP (see also the G_{K1} I-V curve in Figure S1). The AP morphology of the six cell models remains quite different, mainly because of the different degree of rectification (especially for Bett *et al.*¹²) and time dependency (Ishihara *et al.*¹⁹ and O'Hara-Rudy *et al.*⁶), not described by the steady-state I-V curves. On the other hand, the cell models that include I_{K1} according to Dhamoon *et al.*¹⁸ and ten Tusscher *et al.*²¹ – both of them without time dependency – show similar behavior because they have a comparable G_{K1} (see Figure S1B).

Upscaling G_{K1} stabilized RMP towards potentials close to E_K , speeds up the upstroke (higher V_{max}) and hastens the repolarization (shorter APD_{50} and APD_{90}). This behavior is coherent with Meijer van Putten *et al.*¹⁰, who carried out DC experiments scaling the 'electronic' I_{K1} density from 1 to 10 pA/pF. The identification of $G_{K1, critical}$ represents the first step during DC experiments and, using the same I_{K1} formulation, allows the comparison of cells under investigation (since they are in the same state), potentially reducing the intrinsic variability between cells.

The intrinsic variability among cells was computationally investigated by tailoring the hiPSC-CM Paci model in a cell-specific way, according to Lei *et al.*²⁴ Simulations highlighted that variations in $G_{K1, critical}$ between the cell-specific models is small compared to the variation observed between the six different I_{K1} formulations tested. Using $G_{K1, critical}$ to tune DC experiments will lead to a stable and hyperpolarized RMP with reduced variability between cells. Variability of APD and AP waveforms showed in Lei *et al.*²⁴ is preserved, also after the addition of I_{K1} as in our simulations. Variability within the same cell line was shown experimentally by Verkerk *et al.*¹¹, studying hiPSC-CMs with and without I_{K1} addition with DC.

The APD observed in the cell-specific models showed median values comparable to the APD observed by Britton *et al.*²⁸ in the human adult ventricular trabeculae under control conditions (~ 300 ms see Figure 2 in Britton *et al.*²⁸) and by O'Hara-Rudy *et al.*⁶,

who studied small epicardial tissues (~ 280 ms, see Figure 7 in O'Hara-Rudy *et al.*⁶). Except for the models including the Bett I_{K1} formulation, all showed a median APD_{90} value that was higher than the mean value reported by Britton *et al.*²⁸ and O'Hara-Rudy *et al.*⁶. Therefore, upscaling relative to $G_{K1, \text{critical}}$ may be a strategy to make the repolarization faster and to close the gap between *in silico* and experimental data. Upscaling G_{K1} in a range between one and two times the identified critical value brings the AP features close to the experimental range, also in the initial Paci model. The cell-specific models required slightly lower $G_{K1, \text{critical}}$ values to stop the spontaneous activity than the initial Paci model. A difference between the identified values may be explained by the variability between AP waveform in the cell-specific models.

Which I_{K1} formulation to use in DC experiments?

The aim of adding I_{K1} to hiPSC-CMs using the DC technique is to bring about an electrophysiological phenotype that is as close to that of human adult CMs as possible, because this will improve their predictive qualities in drug safety testing. The hyper/depolarizing current prepulse protocol exposed the time-dependent properties of the I_{K1} formulation under investigation. The I_{K1} model based on experimental data from undiseased adult human ventricular cardiomyocytes by O'Hara-Rudy *et al.*⁶ includes an instantaneous rectification factor (R_{K1}, ∞) and an inactivation gating variable (x_{K1}, ∞), described by a first-order kinetics. Despite the theoretical time-dependency, the hiPSC-CM model including the O'Hara-Rudy I_{K1} formulation shows a near perfect overlap between the steady-state I-V curve and the I_{K1} phase plots in all the tested conditions (control, hyperpolarizing and depolarizing prepulse). Closer inspection of the simulation results demonstrated very minimal variation in x_{K1} . This behavior can be explained by the voltage-dependence of the x_{K1}, ∞ parameter for potentials in the AP range. Indeed, for $V_m > -90$ mV, the inactivation variables saturates to 1 (as also described in the original study by O'Hara-Rudy *et al.*⁶, Fig 2B). The minimal variation in the inactivation parameters makes the time-dependency of the O'Hara-Rudy I_{K1} formulation almost negligible (see Figure S4) and explains the overlapping of the I-V curves.

In contrast, the I_{K1} phase plots derived from the simulations using the Ishihara model show clear differences with the I_{K1} steady-state I-V curve. The three I_{K1} phase plots show a substantial current at more positive potentials due to the presence of a transient component. The transient component is determined by the influence of $[Mg^{2+}]_i$ and polyamines (spermine (SPM)_i) and spermidine (SPD)_i) present in cardiomyocytes. The I_{K1} phase plots show various amounts of transient I_{K1} due to the different degree of relief of Mg^{2+} block. The hyperpolarizing prepulse, preceding the

pacing stimulus, opens more channels, which can later become blocked by Mg^{2+} ions at depolarized potentials. The I_{K1} channels blocked at depolarized potentials become again available during repolarization, providing a stronger I_{K1} that shortens the APD. Vice versa, a depolarized prepulse reduces the amount of channels available for the binding with Mg^{2+} ions, leading to a weaker I_{K1} during repolarization and thus a prolonged APD, as previously shown by Ishihara *et al.*¹⁹ employing the comprehensive guinea pig cell model (Kyoto model; see Fig 4C in Ishihara *et al.*¹⁹). The transient component of I_{K1} is an important aspect of I_{K1} as it can contribute to variation in APD and therefore proarrhythmia.

Towards a mature electrophysiological phenotype

The third pillar of the CiPA initiative proposes to employ *in vitro* hiPSC-CMs to confirm the effects of a novel drug predicted by a comprehensive *in silico* model. hiPSC-CMs and adult ventricular CMs show qualitatively consistent responses to some, but not all drugs^{9, 20, 29}. Recently, two computational studies^{8, 30} quantitatively reported on the main electrophysiological differences between hiPSC-CMs and adult ventricular CMs by comparing the Paci 2013 and O'Hara-Rudy models. Paci *et al.*²² investigated the discrepancies between hiPSC-CMs and adult ventricular CMs by simulating the effects of pharmaceutical block of several membrane currents. They observed that the most relevant differences emerged during the block of $I_{Ca,L}$ and I_{K1} , due to the overexpression of I_{NaCa} and a reduced repolarization reserve in hiPSC-CMs. Gong and Sobie³⁰ systematically investigated the differences between hiPSC-CMs and adult ventricular CM models, and designed a mathematical approach to predict the effect of a drug on adult CMs, based on recordings from hiPSC-CMs exposed to the drug. This approach is highly accurate when using *in silico* models; however, its *in vitro* validation is not easy to achieve: the availability of adult ventricular cells is scarce and the variability between hiPSC-CMs is likely to decrease the accuracy.

hiPSC-CMs with a mature electrophysiological phenotype are not available yet. Approaches such as increasing I_{K1} via dynamic clamping, or ectopic overexpression of the *KCNJ2* gene bring the AP waveform closer to that of a human adult cardiomyocyte. Combining this technique with the mathematical methods to extrapolate findings to adult human cardiomyocytes (such as proposed by Gong and Sobie³⁰) may bring us closer to a predictive human cardiomyocyte model.

Conclusion

In this exploratory *in silico* study we addressed the issue of the immature electrophysiological profile of hiPSC-CMs. From the simulations it can be concluded that 1) the electronic expression of I_{K1} according to Ishihara *et al.*¹⁹ is able to successfully stop the automaticity of hiPSC-CMs and shows time-dependent properties that may be important for the evaluation of drug safety, and 2) the definition of $G_{K1, \text{critical}}$ allows researchers to tailor the amount of I_{K1} for each cell, reaching an RMP comparable to adult CMs. $G_{K1, \text{critical}}$ will be a sensitive parameter during *in vitro* DC experiments, a sort of fingerprint that characterizes every cell under investigation. The automatic identification of $G_{K1, \text{critical}}$ will further facilitate implementation of DC on multi-channel automated patch clamp platforms, overcoming the low-throughput that characterizes the combined use of manual patch clamping and DC.

Limitations

The *in silico* results we reported in our work are based on the hiPSC-CM model published by Paci *et al.* in 2013²². More recently, updated or adapted versions of this model were published^{31, 32}. Adoption of these two new hiPSC-CM computational models is likely to lead to slightly different values for $G_{K1, \text{critical}}$ when comparing the six I_{K1} formulations. In this study, we addressed potential hiPSC-CM model dependency of our conclusions by building cell-specific models based on the Paci 2013 model and the approach by Lei *et al.*²⁴ Indeed, the identified $G_{K1, \text{critical}}$ values were slightly different, but still very comparable to the values found using the initial Paci 2013 model. Kernik *et al.*³³ recently published their hiPSC-CM model based on several experimental datasets, collected in different laboratories. Because the model development is different with respect to the aforementioned models, the identification of $G_{K1, \text{critical}}$ in that model would bring further information about model dependency and cellular variability. Furthermore, this study used pacing at 1 Hz, a decision that was informed by our earlier work using human stem cell-derived cardiomyocytes^{34, 35}. Using lower or higher pacing frequencies may affect the observed $G_{K1, \text{critical}}$ values.

Author contributions

AF, TvV, MV and TdB designed the research, AF, BG and TdB performed the research, AF, BG and TdB analyzed the data and AF, BG, MV, TvV and TdB wrote the manuscript.

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Supplementary Material

$G_{K1,original}$		$G_{K1,critical}$	
Model	RMP (mV)	Model	RMP (mV)
Dhamoon	-88.1	O'Hara-Rudy	-86.8
Ishihara	-87.8	Ishihara	-85.9
Bett	-86.8	Bett	-85.9
ten Tusscher	-86.1	ten Tusscher	-83.7
O'Hara-Rudy	-	Dhamoon	-82.6
Paci	-77.2	Paci	-80.4

Figure S1: G_{K1} (V) in original (published) and critical conditions.
(A, B) G_{K1} depends on the membrane potential V_m . The insets magnify the G_{K1} in a voltage range close to the RMP. Larger G_{K1} in this voltage range causes a hyperpolarized RMP, compare the insets and the table.

Figure S2: Comparison of I_{K1} formulations for $G_{K1} = G_{K1, \text{critical}}$
 (A) Action potential waveform and (B) I_{K1} time course in the Paci hiPSC-CM models paced at 1 Hz and with $G_{K1} = G_{K1, \text{critical}}$. Of note, 1) the models including the I_{K1} formulations according to Dhamoon and ten Tusscher (red and cyan traces) show nearly identical membrane potentials and I_{K1} . 2) The low I_{K1} density during the diastolic interval is responsible for a less negative membrane potential in Paci.

Table S1: Cell-specific models: $G_{K1, \text{critical}}$ and average I_{K1} during late repolarization.

Cell ID	Scaling & NaCa	Scaling gKs	Bett		Dhamoon		Ishihara		O'Hara-Rudy		Paci		ten Tusscher	
			Critical GK1	IK1 avg	Critical GK1	IK1 avg	Critical GK1	IK1 avg	Critical GK1	IK1 avg	Critical GK1	IK1 avg	Critical GK1	IK1 avg
			(S/F)	(pA/pF)	(S/F)	(pA/pF)	(S/F)	(pA/pF)	(S/F)	(pA/pF)	(S/F)	(pA/pF)	(S/F)	(pA/pF)
1	3.32	139	599.7	0.746	104.2	0.617	828.7	0.698	249.1	0.720	27.7	0.426	2500	0.590
2	2.08	129	597.4	0.717	104.2	0.593	818.1	0.670	249.2	0.695	27.9	0.414	2506	0.569
3	7.55	125	600.7	0.801	101.6	0.638	831.8	0.735	251.3	0.753	28.0	0.435	2465	0.602
4	21.1	467	597.5	0.916	101.1	0.742	824.9	0.882	253.9	0.988	0.0	0.000	2411	0.692
5	6.89	270	598.6	0.833	103.5	0.690	827.9	0.792	251.3	0.840	25.7	0.432	2470	0.651
6	9.3	211	598.9	0.843	103.6	0.686	830.3	0.793	252.6	0.838	26.3	0.435	2447	0.642
7	0.951	37.6	586.7	0.677	104.0	0.557	826.9	0.634	251.8	0.650	29.8	0.399	2513	0.535
8	1.54	19.5	590.7	0.684	104.5	0.528	828.6	0.610	252.8	0.597	29.2	0.391	2522	0.507
9	1.7	52	591.3	0.694	104.5	0.558	816.4	0.633	251.1	0.644	28.7	0.401	2516	0.535
10	1.67	0	590.9	0.680	104.6	0.480	835.6	0.578	252.9	0.511	29.3	0.379	2527	0.459
11	0.38	31.5	595.4	0.692	104.6	0.594	824.2	0.666	249.1	0.693	32.0	0.435	2539	0.573
12	0.696	18.6	590.2	0.676	104.1	0.558	829.7	0.635	251.0	0.645	30.4	0.401	2520	0.536
13	3.05	153	599.2	0.743	104.0	0.616	828.0	0.698	248.7	0.722	27.6	0.425	2498	0.590
14	1.28	125	589.0	0.693	104.0	0.582	808.6	0.654	249.9	0.688	28.7	0.407	2502	0.557
15	1.19	38.3	585.5	0.680	104.1	0.551	824.9	0.628	252.2	0.639	29.1	0.395	2515	0.529
16	3.02	47.3	600.0	0.718	104.9	0.563	823.0	0.641	249.8	0.637	28.2	0.405	2524	0.539
17	2.35	27	599.3	0.702	105.0	0.537	820.5	0.616	251.5	0.603	28.8	0.398	2528	0.514
18	4.03	138	600.4	0.760	104.3	0.626	828.8	0.709	249.7	0.730	27.5	0.427	2499	0.597
19	5.74	28.9	599.8	0.725	105.3	0.517	826.5	0.597	249.8	0.553	28.2	0.389	2545	0.484
20	2.35	9.18	598.5	0.695	105.0	0.502	827.5	0.591	252.8	0.547	29.0	0.387	2535	0.480
21	3.77	93.7	600.4	0.744	104.5	0.602	828.6	0.683	248.4	0.694	28.1	0.422	2507	0.575
22	13.9	288	599.8	0.883	102.3	0.714	819.9	0.833	251.7	0.904	0.0	0.000	2462	0.673
Mean			595.9	0.741	104.1	0.593	825.4	0.681	250.9	0.695	25.9	0.373	2502	0.565
25th percentile			591.0	0.692	104.0	0.553	823.3	0.629	249.7	0.638	27.6	0.392	2498	0.531
median			598.6	0.717	104.2	0.587	827.2	0.660	251.2	0.690	28.2	0.403	2510	0.563
75th percentile			599.7	0.756	104.6	0.623	828.7	0.706	252.1	0.728	29.1	0.425	2524	0.595
min			585.5	0.676	101.1	0.480	808.6	0.578	248.4	0.511	0.0	0.000	2411	0.459
max			600.7	0.916	105.3	0.742	835.6	0.882	253.9	0.988	32.0	0.435	2545	0.692

We identified $G_{K1, \text{critical}}$ for each cell-specific model ($n=22$) including the six I_{K1} formulations. The results of the bisection search are collected in the $G_{K1, \text{critical}}$ columns. $IK1_{\text{avg}}$ represents the average I_{K1} flowing during the last 50 ms before the next stimulus when the cells are paced at 1 Hz.

Figure S3: Membrane potential and main currents in the cell-specific models.

Membrane potential, I_{K1} , I_{KS} , I_{Kr} , I_{Na} , I_{CaL} and I_{NaCa} of the 22 cell-specific models for each I_{K1} formulation. G_{K1} was set to the critical value identified running the bisection algorithm for each cell model.

Figure S4: Time dependency of I_{K1} in the O'Hara-Rudy formulation. (A-C) Membrane potential, (D-F) I_{K1} density, and (G-I) inactivation variable X_{K1} time courses in CTRL conditions (first column) and in response to depolarizing and hyperpolarizing prepulses (second and third columns, respectively). G-I show the first-order kinetics of X_{K1} , but since it's varying between 1 and 0.993, it can be considered negligible, even in control conditions.

Table S2: Preconditioning prepulse protocol.

APD_{90} in the last AP of a cycle of 30 1 Hz paced APs in CTRL conditions, in response to a depolarizing (DEP) or hyperpolarizing (HYPER) prepulse.

Model	Last APD_{90}				
	CTRL	DEP	$\Delta APD_{90, \text{Dep}}$ (%)	HYPER	$\Delta APD_{90, \text{Hyper}}$ (%)
Bett	237	240	1.3	232	-2.1
Dhamoon	540	544	0.7	522	-3.5
Ishihara	459	489	6.5	384	-16.3
O'Hara-Rudy	678	681	0.4	668	-1.5
Paci	356	357	0.3	339	-4.8
ten Tusscher	545	549	0.7	538	-1.3

Including $G_{K1, critical}$ in an experimental workflow

Figure S5: Including $G_{K1, critical}$ in an experimental workflow.

Proposed steps in dynamic clamp or *in silico* experiments that include determination of $G_{K1, critical}$. (Step 1) Membrane potential is measured from round-shaped and spontaneously beating hiPSC-CMs. (Step 2) *In silico* simulations allow to identify precisely $G_{K1, critical}$ for a cell-specific or an average cell model. In an *in vitro* experiment, the bisection algorithm would take too long, and $G_{K1, critical}$ can instead be estimated with a protocol testing at step-wise increasing or decreasing G_{K1} values. (Step 3) Running subsequent experiments at multiplication of $G_{K1, critical}$ helps to reduce variation between cells. (Step 4) Measuring the AP while a drug is administered gives information about the efficacy or safety of the compound under investigation. Combination with results from a computational model could provide a mechanistic interpretation of what is observed during *in vitro* experiments.

Figure S6: Determining $G_{K1, critical}$ in a dynamic clamp experiment.

(Main figure) In a set of dynamic clamp experiments using the Ishihara formulation, I_{K1} was injected using G_{K1} values starting at 5000 S/F, which was decreased with 500 S/F steps to 0 S/F. $G_{K1, critical}$ was defined as the minimal G_{K1} required for maintaining quiescence, in this example (Cell 2 in inset) $G_{K1, critical}$ was 1000 S/F. (Inset) Summary plot of $G_{K1, critical}$ obtained in 7 cells. In the cells indicated with the arrows, the maximum G_{K1} tested did not completely suppress all activity, i.e. $G_{K1, critical}$ was slightly higher. The average $G_{K1, critical}$ in the 4 cells in which we obtained quiescence was 2750 ± 660 S/F.

$G_{K1, \text{critical}}$ to suppress automaticity of iPSC-CMs

Abstract

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) hold great potential for *in vitro* drug screening. However, iPSC-CMs are considered immature since they lack the inward rectifier current (I_{K1}) and express the nodal funny current (I_f), which in combination cause a depolarized resting membrane potential RMP and automaticity. Overexpression of the KCNJ2 gene, coding for the I_{K1} -channel protein Kir2.1, leads to quiescence after which the cell can only be stimulated by external pacing, which is a rather invasive technique. This can be circumvented by opsins, which are light-sensitive ion channels that can trigger depolarization in response to light, and thereby allow optical pacing. Channelrhodopsin-2 (ChR2) is an opsin, a non-selective ion channel that conducts cations in response to blue light (488 nm), and this has previously been shown to allow optical pacing in iPSC-CMs. Here we co-expressed KCNJ2 and ChR2 in iPSC-CMs to hyperpolarize iPSC-CMs while maintaining excitability through optical pacing. Transfection of KCNJ2 leads to substantial I_{K1} current densities, comparable to adult CMs, and hyperpolarized resting membrane potentials. Action potentials could be evoked by light with different shapes and durations. We observed a high variability between iPSC-CMs and their need for I_{K1} to suppress automaticity while maintaining excitability. In this pilot study we show that iPSC-CMs can be matured in the context of I_{K1} , RMP and suppressing automaticity, but that the introduction of ChR2 does not result in ventricular-like action potentials and raises more questions regarding the electrophysiological consequences.

Introduction

Applying human iPSC-CMs in drug screening or studying the effects of patient-derived gene mutations requires a sufficiently mature electrophysiological phenotype¹. Most importantly, iPSC-CMs should be quiescent and not beat spontaneously. iPSC-CMs beat autonomously because they express the funny current (I_f) and show small densities of the inward rectifier potassium current (I_{K1}). I_f is normally only present in pacemaker cells and I_{K1} is involved in final repolarization of the action potential, thereby maintaining the resting membrane potential in cardiomyocytes¹. iPSC-CMs as a result do not fully repolarize, which elevates the resting membrane potential and lowers the threshold for action potential firing. Recently, I_{K1} levels in iPSC-CMs have artificially been increased with (automated) dynamic clamp^{2, 3} and by overexpression of the gene coding for the I_{K1} channel protein^{4, 5}. Higher levels of I_{K1} lead to more hyperpolarized resting membrane potentials and I_{K1} current densities comparable to adult ventricular cardiomyocytes, which improves the accuracy of detecting QT prolonging drugs⁵. However, strong hyperpolarization also causes quiescence of CMs rendering iPSC-CMs dependent on external pacing for action potential firing⁴. Fine-tuning the KCNJ2 expression levels is essential as well, as there is only a small range where overexpression is functional⁶; too little KCNJ2 does not induce quiescence, whereas too much KCNJ2 results in quiescent but non-excitable iPSC-CMs. Imposing a beating rhythm is vital for development, differentiation and function of cardiomyocytes⁷. Electrical stimulation through pacing is beneficial for drug screening purposes, as the control over beat rate allows the detection of beat-rate dependent effects of certain drugs across a range of frequencies, while reducing variability within and between assays⁸. However, conventional electrical stimulation is rather invasive due to the use of external electrodes, is prone to artefacts and might change the pH of the working solutions through electrolysis⁹. Optical pacing by ChR2 may counteract KCNJ2 overexpression induced quiescence in a non-invasive and biological manner, thereby improving the potential of iPSC-CMs for *in vitro* screening purposes. Channelrhodopsin-2 (ChR2) is a light-gated ion channel that provides an inward flux of cations after stimulation with blue light (wavelength 488 nm), and can be used to depolarize excitable cells¹⁰. The discovery of ChR2 and other opsins have boosted a field named 'optogenetics', which combines optics and genetics to control or monitor the activity of (mostly excitable) tissues¹¹. Optical control has the advantage that it is relatively non-invasive in comparison to regular electrical stimulation. In a proof of concept, embryonic stem cells, that were transfected with ChR2 and then differentiated towards cardiomyocytes (embryonic stem cell-derived cardiomyocytes (ESC-CMs)), showed inward photocurrents in response to

light and responded to optical pacing at different frequencies leading to mechanical contraction¹². Optical pacing by ChR2 has recently been applied to induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) in the context of *in vitro* drug screening with the use of multielectrode arrays (MEAs). Indeed, optical pacing by ChR2 overrides the autonomous rhythm in monolayers of cultured iPSC-CMs and allows pacing at various physiological beating rates, thereby increasing the sensitivity to QT prolonging drugs^{8, 13}. Recently, optical pacing by ChR2 has been combined with simultaneous voltage and calcium read-outs¹⁴ and novel methods to extract single-cell action potentials from field potentials¹⁵. Although optical pacing in a multicellular system improves the drug response of iPSC-CMs, the sensitivity and specificity of the iPSC-CM as an *in vitro* model for drug screening is still severely hampered by the immature electrophysiology^{9, 13}. In this study we examined whether combining overexpression of ChR2 and I_{K1} improved the electrophysiological phenotype of iPSC-CMs. Effective transfection of iPSC-CM was achieved using a method with a lipid/polymer formulation. We observed quiescent CMs after transfection, displaying hyperpolarized resting membrane potentials and higher I_{K1} current densities compared to non-transfected iPSC-CMs. Action potentials could be evoked after stimulation by blue light resulting in varying lengths of action potential duration, indicating a high variability between the amount of I_{K1} needed for suppression of automaticity while maintaining excitability in iPSC-CMs.

Methods

iPSC-CMs

iPSC-CMs were kindly provided by Prof. Zimmermann and Dr. Tiburcy from the Institute of Pharmacology and Toxicology of the University Medical Center of Goettingen, Germany, and Renee Maas MSc from the Laboratory of Experimental Cardiology, Department of Cardiology, University Medical Center Utrecht, the Netherlands. iPSC-CMs were cultured on Matrigel (Corning) coated coverslips in RPMI L-Glutamine medium (ThermoFischer Scientific) with 2% B27 serum replacement (ThermoFischer Scientific) and 1% penicillin/streptomycin (ThermoFischer Scientific).

Transfection

For ChR2 and KCNJ2 delivery to the iPSC-CMs we used *TransIT*®-LT1 transfection reagent (MirusBio) according to manufacturer's instructions. In short, pcDNA3.1-ChR2-EGFP and pcDNA3.1 hs KCNJ2 were added in different ratios to bare RPMI L-Glutamine medium. LT1 was vortexed, added to the RPMI, and the transfection mix was incubated at room temperature for 30 minutes to allow complex formation.

The complexes were added drop-wise to the cells after which the iPSC-CMs were incubated for 48-72 hours at 37°C and 5% CO₂. After incubation, cells were checked for fluorescent signal before further electrophysiological analysis.

Electrophysiology

Whole-cell voltage clamp and current clamp recordings were performed at room temperature (RT) using a HEKA EPC10 USB patch clamp amplifier with Patchmaster software version 2x73.5, or at 37°C using an AxoPatch 200B amplifier controlled by pClamp software version 10.0 (Molecular Devices). Pipettes were pulled with a Sutter P-2000 puller with an average resistance of 2-4 MΩ. The extracellular solution, a modified version of Tyrode's solution, consisted of (in mM): NaCl (130), KCl (4), CaCl₂ (1.8), MgCl₂ (1.2), NaHCO₃ (18), HEPES (10), Glucose (20). The intracellular solution consisted of (in mM): KCl (10), K-Gluconate (125), CaCl₂ (0.6), MgCl₂ (2), HEPES (5), Sucrose (30), Na₂ATP (4), EGTA (5). I_{K1} was measured from a holding potential of -40 mV for 50 ms. Subsequently the cell was clamped at -120 mV and provided with 10 mV increments to +10 mV for 1 s. After that the cell was clamped back at -40 mV for 500 ms. Optical stimulation was applied through the microscope epifluorescence illumination port by a 470 nm LED (Ledxon, blue, 3 W, Conrad Electronic Benelux) at 0.1 mW/mm² with varying stimulus lengths from 5 ms to 100 ms. Electrical stimulation was applied in a frequency of 1 Hz.

Drugs

E-4031 (Sigma-Aldrich) was diluted in H₂O to a stock solution of 1 mM and kept at -20° C. Prior to the experiment, E-4031 was diluted in Tyrode's solution to reach a final concentration of 0.1 mM.

Statistics

All statistical analyses were performed with GraphPad Prism (San Diego, United States of America), version 8 for Mac OS X. Differences between transfected and non-transfected iPSC-CMs were analyzed with an unpaired students t-test or a Mann-Whitney test (in case of non-normality), where $p \leq 0.05$ was considered statistically significant. Values are represented as mean $\pm$ standard error of the mean (SEM).

Results

Transfection of KCNJ2 increases I_{K1} current density and hyperpolarizes iPSC-CMs

iPSC-CMs displayed spontaneous beating behavior within 48-72 hours after thawing. After transfection, iPSC-CMs became quiescent, while spontaneous activity was still

observed in non-transfected iPSC-CMs (Figure 1A). Of note, expression of I_{K1} channels also lowered membrane resistance of iPSC-CMs (from 3.8 ± 1.1 to 1.4 ± 0.2 G Ω at -40 mV), which lowers the ratio between membrane resistance (R_M) and seal resistance (R_{seal}), thereby improving the accuracy of action potential recordings using the patch clamp technique¹⁶.

Figure 1: Effect of transient transfection of ChR2 and KCNJ2 in iPSC-CMs.

iPSC-CMs were transfected with ChR2 and KCNJ2 in a ratio of 2:1 respectively. Action potentials were recorded at room temperature in whole-cell mode. **(A)** Representative trace for untransfected, wild-type iPSC-CMs. **(B)** Representative trace for transfected iPSC-CMs. At * the cell was stimulated by a 488 nm LED for 50 ms.

Overexpression of I_{K1} channels was further studied in voltage clamp experiments (Figure 2). The voltage clamp protocol used to record I_{K1} currents elicited only inward currents in non-transfected iPSC-CMs at potentials below -20 mV. Average steady-state current densities at -120 mV were -34.7 ± 17.0 pA/pF ($n=11$) (Figure 2C). However, in absence of outward currents, the current recorded in non-transfected cells cannot be specified as an I_{K1} current. In contrast, I_{K1} current density was strongly increased in transfected iPSC-CMs, resulting in outward currents at potentials above -60 mV. Average steady-state current at -120 mV was -265.8 ± 35.6 pA/pF ($n=34$), while the average outward current at -50 mV was 9.5 ± 1.4 pA/pF ($n=34$). To further quantify the functional expression of I_{K1} channels after transfection, we determined the maximal conductance of the present I_{K1} channels ($G_{K1,max}$) by fitting the current-voltage relations of each cell between -120 and -90 mV with a linear function. From this we obtained the reversal potential (E_{rev}) and $G_{K1,max}$. Average $G_{K1,max}$ was 5775 ± 3815 pS/pF ($n=29$), average E_{rev} was -66.2 ± 5.0 mV (Figure 2D).

The combined expression of I_{K1} channels and ChR2 was assessed by illumination of the cells with blue light while recording I_{K1} . After switching on the light, an inward current, likely the photocurrent conducted by ChR2, was observed (Figure 2A). By plotting the peak current density of the light-induced current component versus the test potential (Figure 2B), we observed that the ChR2 current was inward at all tested potentials, with an average current density that is comparable to the average outward I_{K1} current density at potentials around the resting membrane potential. The relation between membrane potential and current density of the light-invoked current is very similar to those described earlier for ChR2¹⁷.

ChR2 allows action potential firing by light

I_{K1} -enhanced iPSC-CMs were subjected to varying intervals of light stimulation in order to determine the threshold for optical action potential firing. Initially, KCNJ2 and ChR2 were transfected in a 1:1 ratio in iPSC-CMs, but this did not result in any evoked action potentials by light stimulation, likely due to the overexpressed amount of I_{K1} . However, when we increased the ratio of ChR2 to KCNJ2 to 2:1 we were able to trigger action potentials. Light stimulation pulses differed from 10 and 100 ms between cells. Repetitive light stimulation pulses resulted in consecutive action potentials in some iPSC-CMs enabling optical pacing, but repeated stimulation also resulted in quiescence in other iPSC-CMs. Successfully triggered action potentials at 50% of repolarization (APD_{50}) were 290.7 ± 57.2 ms in transfected iPSC-CMs ($n=7$) compared to 565.5 ± 58.5 ms in wild-type iPSC-CMs ($n=5$) ($p=0.008$) (Figure 3A). Action potentials at 90% of repolarization (APD_{90}) were 358 ± 47.8 ms in transfected iPSC-CMs ($n=7$) compared to 674.4 ± 53.6 ms in wild type iPSC-CMs ($n=5$) ($p=0.001$), indicating that overexpressing I_{K1} in iPSC-CMs resulted in AP shortening (Figure 3B). The average maximal diastolic potential was -40.2 ± 1.1 mV in wild-type iPSC-CMs, which hyperpolarized to a stable resting membrane potential of -71.5 ± 0.3 after transfection ($p=0.0007$) (Figure 3C).

Tailoring the amount of I_{K1} showcases variability in iPSC-CMs

In a following set of experiments, we explored the influence of physiological temperature on the behavior of I_{K1} - and ChR2-transduced iPSC-CMs using a different line of iPSC-CMs. In contrast to previous findings, ChR2:KCNJ2 ratios of 2:1 did not result in any optically triggered action potentials, so we decreased the amount of KCNJ2 in the transfection mix until we were able to trigger optical action potentials. The ratio of ChR2:KCNJ2 was decreased to 15:1, 25:1 and 35:1 after which we were able to trigger optical action potentials.

Figure 2: I_{K1} and ChR2 currents in transfected iPSC-CMs.

iPSC-CMs were transfected with KCNJ2 and ChR2 in a ratio of 1:2 respectively. During a voltage clamp experiment, cells were stimulated with blue light during the last half of each test pulse (indicated with *). **(A)** Representative traces displaying the large inward I_{K1} and light-induced activation of ChR2 currents. **(B)** Current-voltage diagram showing average steady-state I_{K1} current and average peak ChR2 current. **(C)** Current-voltage diagram comparing average steady-state I_{K1} current in transfected and non-transfected cells. **(D)** Plot of data from (C) more closely showing the outward I_{K1} currents at physiologically relevant membrane potentials. **(E)** Relationship between G_{K1} and reversal potential in transfected iPSC-CMs. Crosses depict individual cells, gray ellipse depicts the average G_{K1} and V_{rev} with standard error of the mean (SEM).

Figure 3: Action potential characteristics in transfected iPSC-CMs (RT).

iPSC-CMs were transfected with ChR2 and KCNJ2 in a ratio of 2:1 respectively. Action potentials were recorded at room temperature in whole-cell mode. **(A)** Action potential duration at 50% of repolarization (APD₅₀) for non-transfected (n=5) and transfected (n=7) iPSC-CMs (p=0.008, unpaired t-test). **(B)** Action potential duration at 90% of repolarization (APD₉₀) for non-transfected (n=5) and transfected (n=7) iPSC-CMs (p=0.001, unpaired t-test). **(C)** Resting membrane potential (RMP) for non-transfected (n=5) and transfected (n=7) iPSC-CMs (p=0.0007, unpaired t-test).

Cells that did not respond to light-based activation were paced through current injection at 1 Hz. Non-transfected iPSC-CMs displayed longer action potentials when spontaneously triggered versus electrically paced with APD₉₀s of 189.6 ± 82.1 ms (n=3) in spontaneously beating iPSC-CMs versus 169.9 ± 53.5 ms (n=6) in electrically paced CMs. After transfection with the lowest amount of ChR2 feasible for this study (15:1), APD shortened after optical stimulation to 98 ± 7.7 ms (n=8). However, the transfected iPSC-CMs displayed shorter action potentials when optically paced compared to electrically paced, regardless of ChR2:KCNJ2 ratio. Average APD₉₀ values differed between 36.7 ± 6.5 ms (n=2) in optically paced and 42.6 ± 14.2 ms (n=3) in electrically paced (ratio 20 (ChR2):1 (KCNJ2)). With a higher amount of ChR2, APD₉₀ increased to 51.3 ms (n=1) after optical stimulation, and 93 ± 29.8 (n=7) after electrical stimulation (ratio 25 (ChR2):1 (KCNJ2)). Differences between the optical and electrical stimulation groups became smaller with an even higher amount of ChR2, with APD₉₀ values differing between 54.2 ± 8.9 ms (n=3) and 58.8 ± 28 ms (n=2) respectively (ratio 35 (ChR2):1 (KCNJ2)) (Figure 4A). The resting membrane potential was very depolarized in non-transfected iPSC-CMs with values of -47.1 ± 2.9 (n=3) in spontaneously beating iPSC-CMs and -58.3 ± 3.9 mV (n=6) in electrically paced iPSC-CMs. RMP became more

hyperpolarized with higher amounts of KCNJ2 transfected, suggesting a higher I_{K1} density and indicating the importance of I_{K1} in establishing the RMP (Figure 4B). With the lowest amount of ChR2 and the highest amount of KCNJ2 (15:1 respectively), RMP decreased to -88.6 ± 4.0 mV (n=8). More ChR2 compared to KCNJ2 (20:1) resulted in RMPs of -89.0 ± 3.0 mV (optically paced, n=6) and -85.5 ± 4.0 mV (electrically paced, n=3). An even higher amount of ChR2 resulted in RMPs of -90.2 mV (n=1) in optically paced iPSC-CMs versus -62.8 ± 7.0 mV (n=7) in electrically paced iPSC-CMs. iPSC-CMs transfected with the highest ratio of ChR2 to KCNJ2 (35:1) displayed depolarized RMPs of -46.5 ± 7.3 mV (optically paced) and -57.1 ± 6.7 mV (electrically paced).

Figure 4: Action potential characteristics in transfected iPSC-CMs (37°C).

iPSC-CMs were transfected with different ratios of ChR2 and KCNJ2 and APs were either triggered by light or elicited by current injection, depicted in the figure as 'ratio ChR2:KCNJ2' followed by 'Optical' or 'Electrical'. Action potentials were measured at 36–37°C in whole-cell mode. **(A)** APD₉₀ in iPSC-CMs: non-transfected electrically paced (label 'Control', n=6), 15:1 optical (n=8), 20:1 optical (n=6), 20:1 electrical (n=3), 25:1 optical (n=1), 25:1 electrical (n=7), 35:1 electrical (n=2). **(B)** RMP in the same groups as A.

E-4031 prolongs APD in iPSC-CMs

Previously, I_{K1} -enhanced iPSC-CMs presented prolonged APDs after adding the I_{Kr} blocker E-4031, while non-transfected iPSC-CMs did not display any APD prolongation⁵. In our experiments, 0.1 μ M E-4031 resulted in prolonged action potentials in 5 out of 6 (83%) of the iPSC-CMs measured. Baseline APDs did not differ substantially between different ratios of transfected ChR2 and KCNJ2 with average values of 53.0 ± 2.2 ms, which prolonged to 70.1 ± 10.6 ms after E-4031 addition (Figure 5A). We did observe a high variability in AP shape regarding upstroke, phase 1 repolarization and plateau phase between individual measurements. The upstroke did not reach fully depolarized potentials in some of the action potentials triggered by light (Figure 5D-F) and phase 1 repolarization could only be observed in 50% of the measurements (Figure 5B, C, G).

Discussion

In this study we combined two different ion channels to explore the effect on electrophysiology of iPSC-CMs. We introduced the I_{K1} channel to hyperpolarize the iPSC-CM, and ChR2 to depolarize the iPSC-CM by light. Here we report for the first time a combinational approach to investigate the effects of I_{K1} in combination with optical pacing. The obtained data show hyperpolarization in iPSC-CMs, depending on the amount of transfected KCNJ2, and action potentials triggered by light.

Introducing I_{K1} into iPSC-CMs provides a substantial I_{K1} current density which consequently also lowers the resting membrane potential to values comparable with adult cardiomyocytes. We observed quiescence in transfected iPSC-CMs, which is in line with previous findings^{4,5}. We were able to trigger action potentials by light, although this differed per cell and per cell line, depending on the amount of transfected KCNJ2. CMs with large amounts of transfected KCNJ2 did not respond to optical stimulation or only responded partially with a subthreshold depolarization. The balance between sufficient hyperpolarization and maintaining excitability is very delicate in an iPSC-CM and differs per cell, as previously shown⁶. We used various lines of iPSC-CMs with differing origins (e.g. manner of reprogramming or differentiation protocol), which has an impact on their electrophysiological characteristics. We observed extreme differences in action potential duration at baseline in non-transfected iPSC-CMs originating from different lines, which is a common characteristic for iPSC-CMs¹⁹. The use of these different iPSC-CM lines showcases the variability within iPSC-CMs and illustrates the need for tailored models.

We used the *TransIT*®-LT1 transfection reagent to overexpress the ion channels in the iPSC-CMs. These type of transfection reagents transiently transfect the cell and the success rate depends on the cell type which results in varying efficiency. Furthermore, conventional transfection strategies do not allow the fine control over the amount of channels that are incorporated in the plasma membrane. It therefore complicates determining the threshold of how much I_{K1} is needed to hyperpolarize the membrane potential while maintaining the possibility for excitation. For stable expression of either one of the channels, other methods of transfection can be considered such as adeno-associated viruses (AAVs) to genetically engineer cells and increase the efficiency of transfection^{20, 21}, or optogenetic actuators to generate I_{K1} and create a fully optical system²².

Figure 5: Action potential characteristics in transfected iPSC-CMs after E-4031 addition. iPSC-CMs were transfected with different ratios of ChR2 and KCNJ2 and APs were either triggered by current injection or by light, depicted in the figure as 'Electrical' or 'Optical'. Action potentials were measured at 36–37°C in whole-cell mode. **(A)** APD₉₀ at baseline (black bars) and acutely after 0.1 μM E-4031 addition (green/gray bars). **(B–G)** Representative AP traces of baseline (black) and acutely after 0.1 μM E-4031 addition (green/gray). **(B)** 20:1 electrical (cell 1). **(C)** 20:1 electrical (cell 2). **(D)** 25:1 optical (cell 1). **(E)** 25:1 optical (cell 2). **(F)** 25:1 optical (cell 3). **(G)** 25:1 optical (cell 4).

The potential of incorporating ChR2 in cardiomyocytes for optogenetic pacing has been thoroughly explored, but the light-sensitive channel might also pose some challenges for cardiomyocyte electrophysiology. For example, ChR2 conducts cations selectively, but the resulting photocurrents may contain a combination of cations such as H^+ , Na^+ , Ca^{2+} , and K^+ ¹⁰. In addition to the ion channel properties, ChR2 has been shown to act as an outwardly driven H^+ pump, indicating a dual role in electrophysiology²³. ChR2 is also sensitive to bleaching and desensitization after longer exposure times¹⁰. Indeed, we observed a lack of ChR2 responsiveness after repetitive periods of stimulation in some iPSC-CMs. The use of other optogenetic actuators might be considered for future pacing endeavors, such as the Channelrhodopsin derivative ChRiff which needs 9x less light to be activated²⁴.

This study provided a proof of concept of overexpressing two ion channels in iPSC-CMs in order to evaluate the effects on their electrophysiological characteristics. Due to time constraints, we have not been able to fully explore the effects of the dual expression on the electrophysiology of iPSC-CMs. For example, it will be interesting to evaluate the effects of various ion channel blockers on the iPSC-CM transfected with KCNJ2 and ChR2, such as the I_{CaL} blocker nifedipine, and the I_{K1} blocker barium chloride, to fully evaluate whether adding KCNJ2 will be of additional value for optical pacing using iPSC-CMs in drug screening. This can be evaluated *in silico* using a ChR2 model, which has already been validated for several types of cardiomyocytes. It will be worthwhile to investigate the effect of increasing amounts of ChR2 and/or I_{K1} on iPSC cardiomyocytes on action potential duration and characteristics²⁵.

The advantages of optical pacing over conventional electrical stimulation in the context of *in vitro* screening have been described extensively. Benefits include the non-invasiveness of optical pacing as opposed to electrical pacing using external electrodes. Uniformly distributed optogenetic sensors also allow larger fields of cardiomyocytes to be synchronously activated. Moreover, optical pacing diminishes the use of correction formulas for beating rates, which have not been optimized yet for iPSC-CMs⁸⁻⁹. Furthermore, optogenetic pacing can be applied at lower frequencies than conventional pacing, which increases the sensitivity of iPSC-CMs to drugs that affect CM electrophysiology at low heart rates⁹, although higher pacing rates may be necessary to detect use-dependent block²⁶.

Conclusion

Combining the expression of KCNJ2 and ChR2 in iPSC-CMs leads to hyperpolarized CMs while maintaining excitability with optical pacing. The balance between the amount of KCNJ2 needed and excitability differs per condition which illustrates the previously reported variability within different sources of iPSC-CMs.

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General introduction

Inherited cardiomyopathies represent a group of cardiac diseases in which heart function can become severely compromised. Depending on geographical differences, these diseases affect 1:500 to 1:10'000 individuals worldwide which leads to high mortality¹. Inherited cardiomyopathies are characterized by genetic heterogeneity: they exist in a wide range of phenotypes, each with different penetrance, age of disease onset and track of progression. How the causal mutation actually leads to disease is rather complex and often poorly understood, as not only the mutation but also modifier genes, post-translational modifications, epigenetics and environmental effects can influence the actual phenotype². The presentation of disease may additionally be affected by the body's own compensatory mechanisms, further contributing to the phenotypical heterogeneity of inherited cardiomyopathies.

Development of advanced genomic analyses has improved the detection of aberrancies in genes nowadays associated with inherited cardiomyopathies. Genetic testing does not only aid in diagnostic purposes, it also helps to identify relatives at risk, and guides the choice for appropriate therapeutic interventions³. Furthermore, genomic analyses are envisioned to enable precision medicine, in which patients receive an optimized therapy best suited for their genotype – phenotype combination. In current clinical practice, treatment is diversified not per patient, but per type of cardiomyopathy, and generally consists of lifestyle changes, pharmacological intervention and/or devices such as an implantable cardioverter-defibrillator (ICD)⁴.

The heterogeneous character of inherited cardiomyopathies compromises specific genotype – phenotype predictions, although these are a prerequisite for development of precision medicine. Suitable patient-specific models would be a very helpful tool for studying the effect of mutations and the cardiac-specific effects of drugs, specifically in the context of developing patient-specific therapy. Over the last ten years, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) have been proposed and used as a promising tool to study the mechanism of disease and the effects of drugs *in vitro*. Since its development, a wide range of iPSC-CM lines have been generated in order to mimic various types of inherited cardiomyopathies⁵. Patient-specific iPSC-CMs have become the long-sought tool for patient-specific translational research *in vitro* and are already being used to simulate a clinical trial in a dish⁶. However, the field is so rapidly moving forward that some more introspective studies might be in place in order to verify the true value of these approaches for translational medicine.

In this chapter we examined patient-specific iPSC-CM lines from patients with inherited cardiomyopathies. In Part A we studied a novel mutation in desmoplakin (DSP), a gene associated both with arrhythmogenic and dilated cardiomyopathy (ACM and DCM, respectively). In Part B we studied a mutation in phospholamban (PLN Arg14del) that is involved in the same types of cardiomyopathy, and is a founder mutation in the Netherlands. We analyzed the baseline electrophysiological characteristics and examined the effect of drugs that are either in preclinical test phases or in clinical trials. During these studies we have encountered several practical and inherent limitations of using patient-specific iPSC-CMs for electrophysiological studies. We mirror the iPSC-CM lines used in this chapter to other iPSC-CM lines with a similar mutation, illustrating the variability between iPSC-CM lines. We conclude this chapter with a general advice for patient-specific modeling using iPSC-CMs.

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Introduction

The genetic disorder Arrhythmogenic Cardiomyopathy (ACM) characterizes primarily through thinning of the right ventricular free wall and infiltrating fibro-fatty tissue, predisposing individuals to a high risk on ventricular arrhythmias, heart failure and sudden cardiac death (SCD)¹. ACM, also named Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC/D), encompasses a wide variety of genotypes each resulting in varying clinical pictures, ranging from asymptomatic carriers to severely arrhythmic patients. The disease affects 1:1000 to 1:5000 individuals worldwide, and may present itself for the first time by SCD, particularly in young people and athletes². ACM is a progressive disease, though patients may benefit from treatment options such as implantable cardioverter-defibrillators (ICDs), catheter ablation or anti-arrhythmic drugs³.

ACM is mostly caused by mutations in genes that encode desmosomal proteins and is therefore sometimes referred to as 'a disease of the desmosome'. Desmosomes connect the intermediate filaments of adjacent cells, acting as an anchor for cell-to-cell adhesion. In the heart, desmosomes localize within the intercalated disc where they contribute to withstanding mechanical forces when the heart contracts. Cardiac desmosomes consist of protein networks spanning the intercellular space: the cadherin proteins desmoglein-2 (DSG2) and desmocollin-2 (DSC2) traverse the cell membrane to connect to the armadillo proteins plakoglobin (PKG) and plakophilin-2 (PKP2) inside the cell. PKG and PKP2 then bind to desmoplakin (DSP), which in turn also associates with the intermediate filaments of the cell to secure a tight connection⁴. Mutations in either one of the desmosomal genes may lead to dysfunctional proteins that compromise desmosome integrity. As a result, cardiomyocytes may detach from each other which promotes apoptosis, infiltration of fibrotic cells and fatty deposits².

Desmoplakin is one of the desmosomal proteins that may be affected in ACM. Desmoplakin is the most abundant protein in the desmosome⁵ and mutations in DSP account for 2-12% of the cases of ACM⁶. Beyond deterioration of cardiac function in ACM, patients with a DSP mutation may display aberrant epithelial features such as thickened skin or woolly hair, and those also have a high chance on sudden cardiac death; the best studied examples include Naxos disease and the variation Carvajal syndrome⁷. The mutations in these patients can be characterized as missense or non(mis)sense, resulting in either a truncated DSP protein that cannot interact with other desmosomal proteins or a decreased DSP protein abundance in the desmosome, respectively⁶.

Studying the precise mechanism of the disease is complicated by lack of human tissue, since ACM is usually diagnosed at a progressive stage or post-mortem⁸. As introduced before, the field of disease modeling revolutionized with the development of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Patient-specific iPSC-CMs carry the genetic footprint of their sponsor, also when that sponsor harbors a certain pathogenic mutation, and enable to study the cellular mechanisms from a specific patient. iPSC-CMs can thus potentially be used to examine disease mechanisms or to screen for patient-specific treatment *in vitro*⁹. Furthermore, the pathogenic mutation may be corrected by modifying the affected sequence of an individual by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) enabling patient-specific genome editing¹⁰.

Here we report on a novel mutation in DSP from an individual that met the task force criteria of ACM¹¹. The patient appeared to have a missense mutation (Tyr > His) on codon 1188 of the DSP gene. We created iPSC-CMs from the patients' somatic cells, reverted the mutation by means of CRISPR and characterized the electrophysiological profile of these iPSC-CMs. We also knocked in the patients' mutation into a commercial 'healthy' iPSC line and included a line with a DSP Arg1113X mutation, which is a known pathogenic DSP mutation leading to ACM with a left-dominant phenotype. We characterized the electrophysiological profile of these iPSC-CMs with voltage- and calcium sensitive dyes and compared this to an isogenic control. Finally, we added the sodium channel inhibitor flecainide to modulate the electrophysiological properties of DSP mutated iPSC-CMs.

Methods

iPSC-CM differentiation

Dermal fibroblasts from the patient were obtained following written informed consent. iPSCs were either created from the patients' cells as described in Nishimura *et al.*¹² or derived commercially (ATCC). iPSCs were then differentiated towards the cardiac lineage as described in BurrIDGE *et al.*¹³. We created different lines with a different nomenclature, as depicted below:

Name	Mutation
Tyr1188His	DSP p.Tyr1188His
Reverted	CRISPR-reverted DSP p.Tyr1188His
Arg1113X	DSP p.Arg1113X
Arg1113Xh	DSP p.Arg1113X homozygous
Control	Isogenic control of p.Arg1113X and p.Tyr1188His

Electrophysiology

Cells were seeded on Matrigel coated 10 mm coverslips in a density of 100'000-200'000 per coverslip to allow formation of clusters. Coverslips were incubated for 15 minutes at 37°C with 300 µl complete medium with either 1:1000 FluoVolt™ (ThermoFischer) and 1:1000 Powerload™ (ThermoFischer) or 1:1000 Fluo4AM™ (ThermoFischer). Stock solutions of Fluo4AM™ (1 mM) were prepared using DMSO (Sigma) as solvent. During recordings the coverslips were immersed in a solution containing in mM: NaCl (130), KCl (4), CaCl₂ (1.8), MgCl₂ (1.2), NaHCO₃ (18), HEPES (10) and glucose (10) at 37° C (pH 7.4). Recordings were performed on a custom-built upright microscope (Cairn Research, Kent, United Kingdom) with a 10X objective (Olympus). Blue light was filtered using a 482/35 excitation filter (Semrock FF01-482/35-25) and projected on the objective using a 515 nm dichroic mirror (Chroma T515lp). Fluorescent signal was directed at a 514 long-pass emission filter (Semrock LP02-514RU-25) and captured by a high-speed camera (Andor Zyla 5.5.CL3, Oxford Instruments) at 100 fps for 1000 frames.

Analysis of parameters for voltage and calcium

We used the voltage-sensitive dye FluoVolt™ to record action potentials in iPSC-CM lines. We observed spontaneous beating behavior and did not pace the cells externally. Per coverslip we selected several fields of interest with fluorescent voltage signal. Each field contained 2 to 10 clusters, and n (in numbers) depicts the amount of clusters measured. Recordings were analyzed with a custom-written MATLAB script (DOI 10.17605/OSF.IO/86UFE). From the FluoVolt™ measurements we derived several parameters: action potential duration at 50% and 90% of repolarization (APD₅₀ and APD₉₀ respectively) and the spontaneous beating rate in beats per minute (BPM) (for visualization of the parameters extracted from the data see Figure 1A). The APDs were corrected for the beating rate, following an adjustment of the Fridericia formula used to correct the QT interval for heart rate¹⁴: *BPM corrected APD = APD/(³√(60/BPM))*

From the calcium transient measurements we quantified several features that describe the waveform of the transient: the number of calcium transients per minute, the rise time of the calcium transient (from 10 to 90% of peak amplitude), the amplitude of the transient and the decay time of the transient (from 90 to 10% of peak amplitude), which is the time for reuptake of calcium by Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) into the Sarcoplasmic Reticulum (SR) (for visualization of the parameters extracted from the data see Figure 1B).

Figure 1: Analysis of parameters in this study.

(A) Analysis of action potential characteristics in iPSC-CMs using optical dyes. (B) Analysis of calcium transients in iPSC-CMs using optical dyes.

Statistical analysis

All statistical analysis was performed with GraphPad Prism (San Diego, United States of America), version 8 for Mac OS X. Differences between mutated and reverted lines were analyzed with an unpaired students t-test. Groups with the knocked-in mutations were analyzed using a one-way Analysis of Variance (ANOVA) or a Kruskal-Wallis test with either Tukey's or Dunn's post-hoc test respectively to correct for multiple comparisons within the same group. Groups treated with flecainide were analyzed using a two-way ANOVA and Tukey's test. Data is presented as mean $\pm$ standard error of the mean (SEM) and a p-value of ≤ 0.05 was considered statistically significant.

Results

Patient-specific iPSC-CMs display prolonged APD, which can be reverted by CRISPR

First, we measured the electrical activity in cardiomyocytes differentiated from the iPSCs derived from the patient harboring the DSP Tyr1188His mutation after one month in culture. The mutation was also reverted in an iPSC line from this patient. The reverted line showed a shorter APD_{50} ($p < 0.0001$), APD_{90} ($p = 0.0005$) and a higher beating rate ($p = 0.0002$) compared to Tyr1188His iPSC-CMs (Figure 2). Average APD_{50} values were 294 ± 10 milliseconds (ms) for Tyr1188His and 217 ± 13 ms for reverted, and

APD₉₀ values varied between 339 ± 12 ms for Tyr1188His and 267 ± 16 ms for reverted. Beating rate differed from 56 ± 3 BPM in Tyr1188His to 71 ± 3 BPM in reverted. After two months of differentiation APD₅₀ decreased (from 249 ± 22 ms to 237 ± 11 ms) and BPM increased (from 61 ± 2 BPM to 68 ± 3 BPM) in the reverted lines, which is consistent with the findings in the iPSC-CM line one month after differentiation, though not significant (Figure 3B-D). APD₉₀ again decreased after reverting the mutation in iPSC-CMs (from 331 ± 17 ms to 285 ± 12 ms, $p=0.04$). In the reverted lines we observed an increased expression of the genes coding for the desmosomal proteins plakoglobin, plakophilin-2 and desmoglein-2, the gap junctional protein connexin43 but also for the genes encoding sodium and L-type calcium ion channels (Supplementary Figure 1; for methods see Supplementary Methods).

Figure 2: Action potential characteristics in patient iPSC-CMs after one month of differentiation.

FluoVolt™ was used to assess action potentials in reverted and Tyr1188His iPSC-CMs. **(A)** Representative action potential traces of reverted (left) and Tyr1188His (right) iPSC-CMs. **(B)** Beating rate in BPM in reverted ($n=33$) and Tyr1188His ($n=34$) iPSC-CMs ($p=0.0002$, Mann-Whitney test). **(C)** Action potential duration at 50% of repolarization (APD₅₀) in reverted ($n=33$) and Tyr1188His ($n=34$) iPSC-CMs ($p<0.0001$, Mann-Whitney test). **(D)** Action potential duration at 90% of repolarization (APD₉₀) in reverted ($n=33$) and Tyr1188His ($n=34$) iPSC-CMs ($p=0.0005$, Mann-Whitney test).

Figure 3: Action potential characteristics in patient iPSC-CMs after two months of differentiation.

FluoVolt™ was used to assess action potentials in reverted and Tyr1188His iPSC-CMs. **(A)** Beating rate in reverted (n=10) and Tyr1188His (n=12) iPSC-CMs (p=0.006, unpaired t-test). **(B)** APD₅₀ in reverted (n=9) and Tyr1188His (n=8) iPSC-CMs (p=0.62, unpaired t-test). **(C)** APD₉₀ in reverted (n=9) and Tyr1188His (n=8) iPSC-CMs (p=0.04, unpaired t-test).

Commercial knock-in iPSC-CMs recapitulate the electrophysiological features from the patients' iPSC-CMs

Next we measured commercial iPSC lines differentiated towards the cardiac lineage with a knock-in of either the patient mutation (Tyr1188His), the known pathogenic mutation (Arg1113X), a homozygous variant of the pathogenic mutation (Arg1113X (h)) and an isogenic control (ctrl). Tyr1188His cardiomyocytes beat slower compared to control and Arg1113X cardiomyocytes and presented prolonged APD₅₀ and APD₉₀ (Figure 4). The beating rate of Tyr1188His CMs was around 40 ± 2 BPM, and differed slightly from control which had a beating rate of 43 ± 5 BPM. We observed a prolonged APD₅₀ in Tyr1188His with values of 279 ± 15 ms compared to 207 ± 15 ms in isogenic control (Figure 4C). APD₉₀ was prolonged in the same fashion, with Tyr1188His presenting prolonged APD₉₀ of 452 ± 21 ms compared to 368 ± 19 ms in isogenic control (p=0.03). APDs in the pathogenic DSP mutation (Arg1113X) line did not prolong significantly compared to control, neither in the heterozygous nor in the homozygous line. Beating rate did decrease with 30-40% in the homozygous Arg1113X line compared to other lines (p= 0.02 for ctrl vs Arg1113X (h), p= 0.01 for Tyr1188His vs Arg1113X (h), p=0.05 for Arg1113X vs Arg1113X (h)). Gene expression of the desmosomal proteins desmoglein, plakoglobin and plakophilin was decreased in both mutated lines as well as genes coding for the sodium channel and inward rectifier potassium

(I_{K1}) channel. Mutated CMs further presented a decreased expression of connexin43, similar to the patients' own iPSC-CMs (Supplementary Figure 2).

Figure 4: Action potential characteristics in knock-in iPSC-CMs.

FluoVolt™ was used to assess action potentials in commercial iPSC-derived CM lines in isogenic iPSC-CMs (control), or iPSC-CMs with a Tyr1188His mutation, an Arg1113X mutation or a homozygous Arg1113X mutation (Arg1113Xh). **(A)** Action potential traces of all lines: control (n=19), Tyr1188His (n=62), Arg1113X (n=49) and Arg1113Xh (n=9). **(B)** Beating rate in BPM (p=0.02 between control and Arg1113Xh, p=0.01 between Tyr1188His and Arg1113Xh, p=0.05 between Arg1113X and Arg1113Xh, Kruskal-Wallis test with Dunn's test to correct for multiple comparisons). **(C)** APD₅₀ (p=0.006 between Tyr1188His and Arg1113X, Kruskal-Wallis test with Dunn's test to correct for multiple comparisons). **(D)** APD₉₀ (p=0.03 between control and Tyr1188His, Brown-Forsythe ANOVA with Dunnett's T3 test to correct for multiple comparisons).

Commercial knock-in iPSC-CMs show afterdepolarizations and alternans at baseline

We repeated the measurements in iPSC-CMs obtained from a different differentiation with the same commercial iPSC line and the same clones of both mutated lines, excluding the homozygous Arg1113X line since homozygous mutations have shown to be lethal in animal models¹⁵ and as such do not represent the genotype found in ACM patients. BPM varied between 37 ± 2 for control, 37 ± 1 for Tyr1188His and 35 ± 2 for Arg1113X (Figure 5A). APD_{50} values differed between 347 ± 28 ms for control, and were slightly longer for the mutated lines with 418 ± 20 ms for Arg1113X and 418 ± 23 ms for Tyr1188His (Figure 5B). APD_{90} values were 434 ± 29 ms for control, 525 ± 18 ms for Arg1113X and 498 ± 22 ms for Tyr1188His ($p < 0.0001$ for Arg1113X versus either control or Tyr1188His, Figure 5C). Interestingly, we observed action potential alternans (alternating long and short action potentials) in the control line at baseline. The Tyr1188His cardiomyocytes displayed phase 2 and phase 3 early afterdepolarizations (additional depolarizations during the plateau phase or repolarization respectively) and delayed afterdepolarizations (depolarizations immediately following the previously finished action potential). We did not observe alternans or afterdepolarizations previously in the other lines. Single afterdepolarizations or single events of alternans may not impact action potential formation and propagation, but may become proarrhythmic when occurring frequently¹⁶.

Figure 5: Action potential characteristics in knock-in iPSC-CMs after adding flecainide. FluoVolt™ was used to assess action potentials at baseline (bl) and after the addition of 5 μ M flecainide for 1 minute (F 1 min) and 5 minutes (F 5 min) in commercial iPSC-derived CM lines in isogenic iPSC-CMs (control, n=34 (bl), n=7 (1 min), n=20 (5 min)), or iPSC-CMs with a Tyr1188His mutation (n=38 (bl), n=28 (1 min), n=20 (5 min)) or an Arg1113X mutation (n=70 (bl), n=14 (1 min), n=20 (5 min)). **(A)** Beating rate in BPM in control, Tyr1188His (p=0.0009 between baseline and 1 min, p=0.0065 between baseline and 5 min), and Arg1113X (p=0.01 between baseline and 1 min, p=0.02 between baseline and 5 min). **(B)** APD₅₀ in control, Tyr1188His and Arg1113X (p=0.0002 between baseline and 1 min, p=0.0016 between baseline and 5 min). **(C)** APD₉₀ in control, Tyr1188His (p=0.03 between bl and 5 min), and Arg1113X (p<0.0001 between bl and 1 min, and between bl and 5 min) (p<0.0001 between Arg1113X and control and between Arg1113X and Tyr1188His). A 2way ANOVA with Tukey's test to correct for multiple comparisons was used for all measurements.

Flecainide decreases BPM and increases APD in mutated iPSC-CM lines

Since flecainide has been suggested to have antiarrhythmic properties in combinational treatment for ARVC patients³⁷, we explored the effect of the drug on the APD and BPM of iPSC-CM lines harboring a mutation in DSP. 5 μ M of flecainide was added and the effect on iPSC-CM electrophysiology was recorded after one minute and five minutes exposure. We observed a decrease in BPM in the Tyr1188His line after one minute of flecainide exposure from 37 ± 1 BPM to 31 ± 4 BPM ($p=0.0009$) (Figure 5A), which persisted after five minutes of exposure to 31 ± 2 BPM ($p=0.007$). APD₉₀ prolonged after five minutes of exposure from 498 ± 21 ms in baseline to 618 ± 49 ms ($p=0.003$) (Figure 5C). Flecainide also decreased BPM in Arg1113X lines from 35 ± 2 BPM to 25 ± 2 BPM after one minute ($p=0.01$) and to 27 ± 1 ms after five minutes ($p=0.02$). APD₉₀ prolonged after one minute from 525 ± 18 ms to 805 ± 58 ms ($p<0.0001$) and to 767 ± 24 ms after five minutes ($p<0.0001$).

Calcium transients in iPSC-CMs

Next we analyzed the calcium handling properties of the iPSC-CM lines with the knock-in mutation by means of the calcium-sensitive dye Fluo4AM^{TM}. We observed a higher frequency of calcium transients in mutated lines, indicated by calcium signals per minute (SPM), with an average of 27 ± 3 SPM for Arg1113X, 31 ± 3 SPM for Tyr1188His and 17 ± 1 in control lines ($p<0.0001$ for Arg1113X vs ctrl and for Tyr1188His vs ctrl) (Figure 6A). The rise time of the calcium transient, indicating the speed of the intracellular calcium release, was almost double in the control line compared to the mutated lines, with rise times of 250 ± 10 ms in control lines, 149 ± 6 ms in Arg1113X, and 129 ± 3 ms in Tyr1188His ($p<0.0001$ for both) (Figure 6B). The decay time, signifying the time for intracellular calcium levels to cease, was shorter in Tyr1188His lines compared to either control or Arg1113X lines. Decay time varied from 439 ± 31 ms in Tyr1188His lines to 705 ± 53 ms in control and 667 ± 60 ms in Arg1113X ($p=0.02$ for Arg1113X vs control and $p<0.0001$ for Tyr1188His vs control) (Figure 6C). The amplitude of the calcium transient, indicating the magnitude of intracellular calcium during a single action potential, was similar in isogenic control (646 ± 28 a.u.) and Tyr1188His (626 ± 59 a.u.) but decreased in Arg1113X (295 ± 28 a.u.) ($p<0.0001$ for Arg1113X vs both lines) (Figure 6D). Calcium handling genes such as SERCA2 and RyR2 were downregulated in mutated lines. Together with the electrophysiological findings this suggests less effective calcium handling in DSP mutated iPSC-CMs (Supplementary Figure 2).

Figure 6: Calcium transient properties in knock-in iPSC-CMs after adding flecainide. FluoVolt™ was used to assess action potentials at baseline (bl) and after the addition of 5 μ M flecainide for 1 minute (F 1 min) and 5 minutes (F 5 min) in commercial iPS-derived CM lines in isogenic iPSC-CMs (control, n=18 (bl), n=13 (1 min), n=15 (5 min)), or iPSC-CMs with a Tyr1188His mutation (n=18 (bl), n=20 (1 min), n=18 (5 min)) or an Arg1113X mutation (n=19 (bl), n=21 (1 min), n=18 (5 min)). **(A)** Amount of signals per minute (SPM) ($p<0.0001$ between control and Tyr1188His and between control and Arg1113X). **(B)** Rise time ($p<0.0001$ between control and Tyr1188His and between control and Arg1113X). **(C)** Decay time for control, Tyr1188His ($p=0.0003$ between bl and 1 min, $p=0.005$ between bl and 5 min) and Arg1113X ($p<0.0001$ between bl and 1 min and between bl and 5 min). **(D)** Transient amplitude ($p<0.0001$ between control and Tyr1188His and between Arg1113X and Tyr1188His). A 2way ANOVA with Tukey's test to correct for multiple comparisons was used for all measurements. **E:** Representative traces of the calcium transients in baseline condition.

Flecainide prolongs calcium transient decay time in iPSC-CMs

We also added 5 μ M flecainide to observe the effect on calcium handling in mutated and control iPSC-CMs after one minute and five minutes of exposure. Signals per minute, rise time or transient amplitude did not change in either one of the lines. The decay time did increase in both mutated lines, from 666 ± 60 ms to 1144 ± 64 ms in Arg1113X after one minute to 1186 ± 124 ms after five minutes of exposure, and increased in the Tyr1188His line from 439 ± 31 ms to 861 ± 63 ms after one minute to 784 ± 57 ms after five minutes of exposure (Figure 6). This is consistent with the finding that flecainide addition prolongs APD in mutated iPSC-CMs. Flecainide targets mainly sodium channels, but may also activate the reverse-mode of the sodium-calcium exchanger (NCX) causing an influx of calcium into the cardiomyocyte. The prolonged calcium transients, with slow decay times, suggest a sustained calcium activation likely due to NCX activity^{18,19}. Additionally, flecainide has been shown to target RyR2, leading to prolonged calcium transients²⁰.

Discussion

In this chapter we describe for the first time the electrophysiology and molecular biology in iPSC-CMs from a newly identified mutation in DSP from an ACM patient. The mutation results in altered cardiac electrophysiology, as we observed prolonged action potentials both in the patients' own iPSC-CMs as well as in the commercial iPSC lines where the mutation was introduced via CRISPR-Cas9. Prolonged APD is a hallmark of ACM, likely due to desmosomal disruption in the intercalated disc leading to improper action potential formation and propagation. Indeed, mutated iPSC-CMs present lower expression levels of desmosomal genes and genes encoding ion channel proteins. Mutated iPSC-CMs further display more calcium signals per minute with short rise and decay times but with an amplitude comparable to control iPSC-CMs. Reverting the DSP mutation by CRISPR resulted in shorter APDs. The increase in expression of genes coding for desmosomal proteins and gap junctional protein connexin43 in reverted and isogenic control suggests increased stability of the desmosome and the intercalated disc. Increased stability could promote trafficking of ion channels to the cell membrane thus facilitating more effective action potential formation and propagation.

iPSC-CMs with a desmoplakin mutation (p. Arg451Gly) have been studied before, though these iPSC-CMs did not show any electrical abnormalities in contrast to the ACM patients carrying the mutation²¹. iPSC-CMs have been studied before from ARVC/D patients with a mutation in PKP2, which is one of the other desmosomal

proteins being most frequently mutated in ARVC/D. These iPSC-CMs expressed less PKP2 and plakoglobin, but expression of desmoplakin and connexin43 did not differ between mutated and control iPSC-CMs. Localization of DSP and Cx43 also did not change in iPSC-CMs with mutated PKP2²². In a different study with PKP2 depleted iPSC-CMs plakoglobin was found to translocate to the nucleus. Compared to control, PKP2 knock-out iPSC-CMs showed longer APD₉₀s compared to control, as well as longer relaxation times, indicating abnormalities with intracellular calcium handling⁵. Microelectrode Array (MEA) measurements in a different iPSC-CM line with a PKP2 mutation showed prolonged rise times in the field potential²³. We did observe longer APD₉₀s as well, but no prolonged calcium transient decay times.

The most common DSP mutation in arrhythmogenic cardiomyopathies is the Arg1113X mutation, which is a known pathogenic mutation leading to a premature stop codon in the DSP protein and is associated with left-dominant arrhythmogenic cardiomyopathy. Mouse models of this DSP mutation display overt structural characteristics similar to ACM patients, such as CM apoptosis, fibrosis, and fatty deposits^{15, 24} and suffer from ventricular arrhythmias¹⁵. Inside the cell, desmosomal proteins aggregated at the intercalated disc while intermediate filaments and microtubules appeared disrupted²⁵. We included this pathogenic DSP mutation in our iPSC-CM study, though we focused on electrophysiology and not specifically on structural characteristics. Our iPSC-CMs showed APD prolongation, which could be a substrate for proarrhythmia, but we did not observe any afterdepolarizations or other markers further supporting that hypothesis.

The measurements in the iPSC-CMs with the novel DSP mutation showed multiple similarities with iPSC-CMs harboring the known pathogenic DSP mutation but also notable differences. For example, the Arg1113X line showed smaller calcium transient amplitudes compared to either Tyr1188His or control. Smaller calcium transients indicate aberrances in intracellular calcium cycling, either through inefficient calcium loading, ryanodine receptor malfunctioning or reduced SERCA activity²⁶. However, gene expression levels for calcium handling genes were not different between the two mutated lines. The aberrant calcium handling might also partially be explained by the electrophysiological nature of iPSC-CMs. iPSC-CMs are known for their underdeveloped SR, lack of T-tubules and reduced sodium channel activity as a result of a depolarized resting membrane potential due to low levels of I_{K1} ²⁷.

We added 5 μ M flecainide to control and mutated iPSC-CM lines and observed the effects on APD and beating rate. Beating rate slowed down and action potentials

prolonged in the presence of flecainide. Also, the calcium transient decay time increased after flecainide addition. Flecainide blocks sodium channels and is a class Ic anti-arrhythmic that is used to treat tachycardias such as atrial fibrillation. Flecainide may, apart from sodium channels, also influence NCX and activate reverse mode, promoting calcium influx. Previously, flecainide reduced triggered activity and delayed afterdepolarizations in iPSC-CMs from patients with inherited cardiac syndromes^{18, 20, 28}. The drug is currently being tested in a randomized double-blind clinical trial pilot in ARVD patients with an ICD to reduce the amount of ectopic ventricular beats in these patients²⁹.

We observed large variability between iPSC-CM lines, even between lines that originated from the same commercial iPS line with the same clone. We observed afterdepolarizations and alternans in one iPSC-CM line at baseline, which we did not observe in the other iPSC-CM line with the same iPSCs, the same clone and the same differentiation method. Large variability remains a problem within the field of iPS disease modeling hampering applications for *in vitro* practices. iPSCs originate from different patients and may thus carry single-nucleotide variants or epigenetic signatures that contribute to variability³⁰. Furthermore, iPSCs need to withstand multiple handling procedures in culture (expansion, gene editing, differentiation) further contributing to the heterogeneity and technical variation³¹. To increase the prospective value of the DSP iPSC-CM model, one should increase the amount of differentiations and the amount of clones used to create more biological replicas. In this way, the iPSC-CM will fully cover the biological profile of the mutation.

In this study we optically measured the electrical activity of cardiomyocyte clusters seeded on coverslips in 2D, which brings some limitations regarding the relevance of *in vitro* systems to study disease mechanisms. 2D cultures with clusters of connected ventricular cardiomyocytes provide some insight into cell-cell connection, action potential initiation and propagation but naturally do not capture the full interplay of cardiomyocytes and non-cardiomyocytes such as fibroblasts and endothelial cells that is necessary for cardiac homeostasis. To mimic ACM *in vitro*, ideally models should include 3D cultures with different cell types that allow structural changes to take place, given that ACM is a structural disease that progresses over time.

Another limitation is that we have used dyes to measure the electrical activity in iPSC-CMs. Though this provides relevant information on AP characteristics and calcium transients, it lacks the quantification of specific ion current densities or membrane potentials and fails to capture AP characteristics such as upstroke velocity. For

this, golden standard assays as single-cell patch clamp or recordings using sharp microelectrodes may have additional value.

Conclusion

In this study we have demonstrated the potency of using patient-specific iPSC-CMs for disease modeling *in vitro*. This model proved to be valuable in exploring the cellular mechanisms underlying action potential generation and calcium handling. Possibly, this model may be used in the future to screen for suitable (previously approved) drugs to bring precision medicine one step closer. However, the relatively immature electrophysiology of iPSC-CMs might influence drug response and hamper the applicability for disease modeling *in vitro*.

Author contributions

BG, SvK, TdB, EvR and TVV designed the study. BG and SvK conducted experiments and analyzed the data (SvK: iPSC-CM differentiation and PCR, BG: electrophysiology). BG wrote the manuscript, TdB and TvV reviewed and edited the manuscript.

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Supplementary Material

Supplementary Methods

Quantitative Polymerase Chain Reaction (PCR)

Total RNA was extracted from hiPSC-derived cardiomyocytes using the RNeasy Mini Kit (Qiagen, 74104) according to the protocol provided by the manufacturer. RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, 1708891). For qPCRs, the BioRad CFX96 Real-Time PCR Detection System and the iQ™ SYBR® Green Supermix kit (BioRad, 170-8885) were used. Relative gene expression between conditions was determined with the comparative CT method, using *HARP* as housekeeping gene.

Name	Forward primer (5'→3')	Reverse primer (5'→3')
DSP	GCACCAGCAGGATGTACTATT	TCAATTCAGGCTGCACGAT
DSC2	TGGTAGAGTTAACCTGAAAGAGTG	TGGTTCTCAGTGTTGGAAAGT
DSG2	GGAACACAGCAGCTACACTT	ACCATCCCTTCAAGCACTTTAT
JUP	ACCAGGAGAGCAAGCTGAT	CTCCACAATGGCAGGCTTATT
PKP2	TGCTAAAGGCTGGCACAA	TAATCGCTGTGCGTGTAGTG
SERCA2	ACCCACATTTCGAGTTGGAAG	CCAACGAAGGTCAGATTGGT
RYR2	GCTGGTTGTGGACTGCAAAG	AACACTCAGGCCTCCGAATG
CACNA1C	GCCTTCAAACCCAAGGGTTACT	TGCTGCCAATTACGATGAGGA
SCN5A	TCTCTATGGCAATCCACCCCA	GAGGACATACAAGGCGTTGGT
GJA1	TTAAGGATCGGGTTAAGGGAAAG	TGTACCCAGGAGGAGACATAG
HARP	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAGGAGAAG

Supplementary Figure 1: Gene expression levels in patient and reverted iPSC-CMs.

Gene expression levels as determined by qPCR. The bars for the mutated lines represent normalized values against control. Amount of samples described as (n=reverted, Tyr118His). (A) Desmoplakin (n=9, 7). (B) Desmocollin-2 (n=8, 7). (C) Desmoglein-2 (n=9, 7). (D) Plakoglobin (n=9, 7). (E) Plakophilin-2 (n=9, 7). (F) Sarcoplasmic/endoplasmic reticulum calcium ATPase (n=8, 7). (G) Ryanodine Receptor-2 (n=6, 6). (H) Gene coding for the L-type calcium channel protein (n=6, 6). (I) Gene coding for the sodium channel protein (n=6, 6). (J) Connexin43 (n=8, 6). Unpaired t-test or Mann-Whitney test.

Supplementary Figure 2: Gene expression levels in knock-in and isogenic control iPSC-CMs.

Gene expression levels as determined by qPCR. The bars for the mutated lines represent normalized values against control. Amount of samples described as (n=reverted, Tyrl188His, Arg1113X) (A) Desmoplakin (n=12, 12, 12), (B) Desmoglein-2 (n=12, 12, 12), (C) Desmoglein-2 (n=12, 12, 12), (D) Plakoglobin (n=12, 11, 12), (E) Plakophilin-2 (n=12, 12, 12), (F) Sarcoplasmic/endoplasmic reticulum calcium ATPase (n=12, 12, 12), (G) Ryanodine Receptor-2 (n=8, 8, 8), (H) Gene coding for the L-type calcium channel protein (n=8, 8, 8), (I) Gene coding for the sodium channel protein (n=12, 12, 12), (J) Connexin43 (n=4, 4). One-way ANOVA or Kruskal-Wallis test.

Introduction

Abnormal calcium handling has been shown to contribute to arrhythmogenesis in various cardiac diseases, ranging from genetic syndromes to acquired disorders¹. Under physiological conditions, after a cardiomyocyte depolarizes, calcium (I_{Ca}) flows into the cell through opening of L-type calcium channels. I_{Ca} then promotes the subsequent opening of Ryanodine Receptors (RyR) in the Sarcoplasmic Reticulum (SR), causing a bulk release of additional calcium ions which in turn activate the myofilaments to contract. After contraction, calcium is pumped back into the SR by the Sarcoplasmic/Endoplasmic Reticulum ATPase (SERCA; SERCA2a in cardiomyocytes) and extruded out of the cell via the sodium-calcium exchanger (NCX)². The force of contractility depends on the magnitude of the calcium store inside the cardiomyocyte and the balance between calcium load and re-uptake which is mediated by different regulators³.

Phospholamban (PLN) is one of those regulators. PLN is a 52 amino acid protein that binds to SERCA and acts as an inhibitor by decreasing the affinity of SERCA for calcium. After β -adrenergic stimulation, PLN can be phosphorylated by either protein kinase A (PKA) or Calcium-calmodulin dependent protein kinase II (CaMKII) which relieves the inhibition of SERCA, allowing a more pronounced re-uptake of calcium by the SR through SERCA⁴. PLN can be dephosphorylated by Protein Phosphatase 1 (PP1), which also inhibits SERCA2a function, or inhibited by HS-1 associated protein X-1 (HAX1) which binds to PLN phosphorylation sites⁵. Because of its dual role in cardiac contractility, PLN is often referred to as a reversible inhibitor.

Overexpression of PLN has been shown to cause superinhibition of SERCA, causing decreased calcium uptake, higher cytosolic calcium levels, decreased calcium release, ultimately leading to impaired contractile performance⁶. Not only overexpression, but also dysfunctional PLN may lead to aberrant calcium dynamics and has been associated with different arrhythmogenic cardiomyopathies, such as Arrhythmogenic Cardiomyopathy (ACM), also known as Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC/D), Dilated Cardiomyopathy (DCM), and Hypertrophic Cardiomyopathy (HCM)⁷. PLN p.Arg14del, hereafter named 'Arg14del', is one of the mutations contributing to an arrhythmogenic phenotype. Arg14del has been identified in both ARVC and DCM patients with a founder mutation in the Netherlands, and is associated with biventricular cardiomyopathy and interstitial fibrosis⁸. Patients may suffer from life-threatening ventricular arrhythmias and a high

risk on sudden cardiac death (SCD) and as such may benefit from an implantable cardioverter-defibrillator (ICD) or a heart transplantation when available⁹.

Istaroxime, a drug known to block the Na⁺/K⁺-transporting ATPase, has previously been identified as a SERCA stimulator¹⁰ and has been shown to enhance calcium dynamics and contraction in various animal models. Istaroxime increased calcium-induced calcium release, SR calcium store and re-uptake of calcium by the SR probably by relieving PLN inhibition^{11, 12, 13}. More recently, istaroxime was also applied in a canine model of chronic atrio-ventricular block where it shortened action potential duration and did not evoke arrhythmic episodes¹⁴. Because of the SERCA-activating, lusotropic (promoting relaxation) and inotropic (promoting contraction) properties of istaroxime, the compound may ameliorate aberrant calcium handling in the PLN Arg14del arrhythmic phenotype.

Here, we studied human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) from a patient with the PLN Arg14del mutation, with and without the presence of istaroxime, and characterized the electrophysiological characteristics using voltage-sensitive and calcium-sensitive dyes.

Methods

iPSC-CMs

Biological material was obtained from a 55-year old male DCM patient harboring a PLN Arg14del mutation after receiving written consent. Dermal fibroblasts were reprogrammed to iPSCs and differentiated towards the cardiac lineage following a protocol adapted from Lian *et al.*¹⁵.

Electrophysiology

Cells were seeded on Matrigel coated 10 mm coverslips in a density of 100'000-200'000 per coverslip to allow formation of clusters. For action potential recordings, coverslips were incubated for 15 minutes at 37°C with 300 µl complete medium with 1:1000 FluoVolt™ (ThermoFischer) and 1:1000 Powerload™ (ThermoFischer). For calcium transient recordings, coverslips were incubated for 60 minutes at 37°C in 1 ml FluoroBrite™ DMEM media (ThermoFischer) containing 0.02% Pluronic® F-127 (Sigma) and 1.25 mM Cal-520® AM Calcium Dye (Abcam). During recordings the coverslips were immersed in a solution containing in mM: NaCl (130), KCl (4), CaCl₂ (1.8), MgCl₂ (1.2), NaHCO₃ (18), HEPES (10) and glucose (10) at 37° C (pH 7.4). Recordings were performed on a custom-built upright microscope (Cairn Research,

Kent, United Kingdom) with a 10X objective (Olympus). Blue light was filtered using a 482/35 excitation filter (Semrock FF01-482/35-25) and projected on the objective using a 515 nm dichroic mirror (Chroma T515lp). Fluorescent signal was directed at a 514 long-pass emission filter (Semrock LP02-514RU-25) and captured by a high-speed camera (Andor Zyla 5.5.CL3, Oxford Instruments) at 100 fps for 1000 frames.

Istaroxime

Stock solutions of 100 mM istaroxime (MedChemExpress) were prepared in DMSO (Sigma) and diluted in the recording bath solution to either 1, 3 or 10 μ M. Recordings were performed after one and five minutes of incubation.

Analysis of parameters for voltage and calcium

We used the voltage-sensitive dye FluoVolt™ to record action potentials in iPSC-CM lines. We observed spontaneous beating behavior and did not pace the cells externally. Per coverslip we selected several fields of interest with fluorescent voltage signal. Each field contained 2 to 10 clusters, and n (in numbers) depict the amount of clusters measured. Recordings were analyzed with a custom-written MATLAB script (DOI 10.17605/OSF.IO/86UFE). From the FluoVolt™ measurements we derived several parameters: action potential duration at 50% and 90% of repolarization (APD_{50} and APD_{90} respectively) and the spontaneous beating rate in beats per minute (BPM). The APD s were corrected for the beating rate, following an adjustment of the Fridericia formula used to correct the QT interval for heart rate¹⁶: $BPM \text{ corrected } APD = APD / (\sqrt[3]{60/BPM})$

From the calcium transient measurements we quantified several features that describe the waveform of the transient: the number of calcium transients per minute, the rise time of the calcium transient (from 10 to 90% of peak amplitude), the amplitude of the transient and the decay time of the transient (from 90 to 10% of peak amplitude), which is the time for reuptake of calcium by Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) into the Sarcoplasmic Reticulum (SR).

Statistics

All statistical analysis was performed with GraphPad Prism (San Diego, United States of America), version 8 for Mac OS X. Differences between mutated and control lines were analyzed with an unpaired students t-test or a Mann-Whitney test. Groups treated with istaroxime were compared to control and analyzed using a one-way Analysis of Variance (ANOVA) or a Kruskal-Wallis test with Dunnett or Dunn's post-hoc test respectively to correct for multiple comparisons within the same group. Data is

presented as mean $\pm$ standard error of the mean (SEM) and a p-value of ≤ 0.05 was considered statistically significant.

Results

Aberrant calcium handling in PLN iPSC-CMs

We used the calcium-sensitive dye Cal-520® AM to visualize calcium transients 40 days post differentiation. In the PLN line we observed a lower frequency of calcium signals per minute (SPM) compared to control, with an average of 37 ± 4 , compared to 75 ± 3 respectively ($p < 0.0001$, Figure 7B). Calcium transient rise time was longer in PLN iPSC-CMs with 137 ± 10 ms compared to control 94 ± 6 ms ($p = 0.0007$, Figure 7C). Decay time was prolonged as well, with PLN presenting 517 ± 31 ms while control presented 343 ± 20 ms ($p < 0.0001$, Figure 7D). Calcium transient amplitudes were decreased in PLN: 1079 ± 126 a.u. in PLN compared to 1393 ± 131 a.u. in control (Figure 7E).

We repeated the same measurements in PLN and control lines 60 days post differentiation to assess whether prolonged culture would influence the calcium dynamics (Figure 8A-D). We observed a similar trend after 60 days post differentiation compared to 40 days post differentiation, with the PLN line firing less signals per minute ($p < 0.0001$), prolonged calcium transient rise times ($p = 0.02$) and decay times ($p < 0.0001$) and decreased calcium transient amplitudes ($p = 0.0001$).

Istaroxime decreases rise time in Arg14del iPSC-CMs

Subsequently, in a next set of experiments we applied istaroxime to PLN iPSC-CMs at 1 and 3 μ M and examined the effects on the calcium dynamics. Istaroxime did not affect calcium dynamics greatly as it only significantly decreased the amount of signals per minute from 45 ± 2 at baseline to 31 ± 1 after 1 μ M and 31 ± 2 after 3 μ M ($p = 0.004$ and $p = 0.001$ respectively), and reduced the rise time from 129.5 ± 10.3 ms at baseline to 96.0 ± 6.5 ms after 1 μ M ($p = 0.02$) (Figure 9). We applied istaroxime to wild-type iPSC-CMs as well at 1, 3 and 10 μ M and observed the effects on the calcium handling after one minute and five minutes. Istaroxime only slightly increased the SPM and the rise time at 1 μ M (Figure 10). We did observe a shift in the activation pattern with higher doses of istaroxime or longer incubation time. Under physiological conditions, calcium release is fast and synchronous throughout the cell.

Figure 7: Calcium handling in PLN iPSC-CMs 40 days after differentiation. Cal520 was used to measure calcium transients in control (n=17) and PLN (n=9) iPSC-CMs. **(A)** Traces of the calcium transient in control and PLN iPSC-CMs. **(B)** Amount of signals per minute (SPM) ($p < 0.0001$, Mann-Whitney test). **(C)** Rise time ($p = 0.0007$, unpaired t-test). **(D)** Decay time ($p < 0.0001$, unpaired t-test). **(E)** Transient amplitude.

Figure 8: Calcium handling in PLN iPSC-CMs 60 days after differentiation.

Cal520 was used to measure calcium transients in control ($n=7$) and PLN ($n=33$) iPSC-CMs. **(A)** Amount of signals per minute ($p<0.0001$, Mann-Whitney test). **(B)** Rise time ($p=0.02$, unpaired t-test). **(C)** Decay time ($p<0.0001$, Mann-Whitney test). **(D)** Transient amplitude ($p=0.0001$, Mann-Whitney test).

However, after applying higher doses of istaroxime or longer exposure time, this synchronous intracellular calcium release was hindered. Some cardiomyocytes displayed fast re-entrant intracellular calcium waves, while in other cardiomyocytes calcium transients broke into smaller sparks. Cardiomyocytes with aberrant calcium handling did not contract anymore and signals from the calcium recordings were highly irregular (Figure 10E).

Figure 9: Calcium handling in PLN iPSC-CMs after addition of istaroxime. Cal520 was used to measure calcium transients at baseline (bl, n=9), after 1 μM of istaroxime (n=11) and 3 μM of istaroxime (n=9) in PLN iPSC-CMs. **(A)** SPM (p=0.0004 between bl and 1 μM and p=0.001 between bl and 3 μM , Kruskal-Wallis test). **(B)** Rise time (p=0.02 between bl and 1 μM , ANOVA). **(C)** Decay time. **(D)** Transient amplitude.

Figure 10: Calcium handling in wild-type iPSC-CMs after addition of istaroxime.

Cal520 was used to measure calcium transients at baseline (bl, n=45), after 1 μ M (1 min, n=18 and 5 min, n=18) or 3 μ M of istaroxime (1 min, n=9 and 5 min, n=4) in control iPSC-CMs. **(A)** SPM ($p=0.0002$ between bl and 1 μ M 1 min, ANOVA with Dunnett's test to correct for multiple comparisons). **(B)** Rise time ($p=0.002$ between bl and 1 μ M 5 min, Kruskal-Wallis test with Dunn's test to correct for multiple comparisons). **(C)** Decay time. **(D)** Transient amplitude. **(E)** Traces of calcium transients after 5 min of 3 μ M istaroxime in control iPSC-CMs.

Discussion

PLN is an important regulator of cardiac calcium homeostasis through reversible inhibition of SERCA. The PLN Arg14del mutation is associated with different types of cardiomyopathies such as DCM and ACM, where patients may suffer from contractile dysfunction, an ejection fraction of <45% and a high risk on ventricular arrhythmias and heart failure^{8,17}. In this study we describe the electrophysiological characteristics of PLN Arg14del iPSC-CMs using voltage-sensitive and calcium-sensitive dyes and compare them to healthy iPSC-CMs with wild-type PLN. We observed short action potentials and aberrant calcium dynamics in PLN Arg14del iPSC-CMs, similar to previous findings¹⁸. In our study, PLN Arg14del iPSC-CMs exhibited fewer calcium transients per minute, longer calcium transient rise and decay times and smaller calcium transient amplitudes compared to control iPSC-CMs. Higher cytosolic calcium levels or depressed reuptake, as indicated by the prolonged calcium transient decay time, can make the cardiomyocyte susceptible to proarrhythmia¹⁹, which correlates well with the arrhythmogenic phenotype of Arg14del patients.

In previous studies using iPSC-CMs, Transcription Activator-Like Effector Nucleases (TALENs) were employed to correct the PLN mutation in iPSC-CMs. 'Corrected' iPSC-CMs displayed longer action potentials and a lower beating frequency compared to the PLN Arg14del iPSC-CMs and did not display any irregular calcium transients¹⁸. Introducing PLN via adeno-associated viral expression in a DCM iPS model increased calcium transient amplitude and calcium transient decay time²⁰. Arg14del iPSC-CMs have also been studied in engineered heart tissues (EHTs). EHTs are microtissues containing cardiomyocytes and/or non-cardiomyocytes seeded in a biomimetic mold that can be subjected to a mechanical load. EHTs have been generated from iPSC-CMs with the PLN Arg14del mutation, the corrected iPSC-CMs and iPSC-CMs from a healthy patient. PLN Arg14del EHTs had a higher spontaneous beating rate, but lower twitch force generation, which could be ameliorated by correcting for the mutation. The twitch force generation was even higher in corrected EHTs compared to the healthy EHTs²¹.

Istaroxime has been applied before to wild-type iPSC-CMs and L-type calcium was measured using whole cell patch clamp. Istaroxime accelerated calcium transient decay but did not influence other calcium transient characteristics²². In our study, the amount of calcium signals per minute decreased in patient iPSC-CMs, while it did not change calcium transient dynamics significantly in control. As a SERCA activator we expected the calcium transient decay times to decrease after addition

of istaroxime through improved SR function. Istaroxime has been applied before in a zebrafish model of the Arg14del mutation, where it ameliorated PLN-induced calcium abnormalities by decreasing the rise and decay time of the calcium transient, increasing the calcium transient amplitude and increasing contractility through a higher ejection fraction and a higher end diastolic volume²³. The improved contractile parameters were also observed in the wild-type zebrafish hearts after addition of istaroxime. Action potential duration shortened and action potential alternans decreased after istaroxime treatment. In wild-type iPSC-CMs we observed spiraling intracellular calcium waves after adding istaroxime, which impacted contraction. Likely, the addition of istaroxime causes hyperactivation of SERCA, increasing the speed of calcium recycling within the cardiomyocyte to an extent where the cardiomyocyte cannot contract anymore. Spiraling calcium waves can result in re-entry excitation which in turn can lead to fibrillation or arrhythmia²⁴.

This study has a number of limitations. First, we compared the PLN Arg14del iPSC-CMs to a healthy control, which was not genetically matched to the patient (isogenic control not available). Using a wild-type control might be a large confounder in this study, as there will be genetic variation between the patient and the control and the electrophysiological differences that we observed may not necessarily be caused by the mutation rather than by sheer biological variability²⁵. One of the other limitations is the immature calcium handling of iPSC-CMs. iPSC-CMs express some components of the calcium machinery such as L-type calcium channels, the sodium-calcium exchanger (NCX), and have an underdeveloped SR²⁶. iPSC-CMs further lack a T-tubule network and as a consequence display poor co-localization of calcium channels and ryanodine receptors²⁷. Therefore, the calcium release is slower, less organized and efficient in iPSC-CMs compared to adult cardiomyocytes. This should be taken into account when studying calcium-related cardiomyopathies in iPSC-CMs.

Conclusion

In this study we explored the electrophysiological characteristics of patient-specific PLN Arg14del iPSC-CMs. Patient-specific PLN iPSC-CMs generally recapitulate calcium abnormalities in line with the clinical phenotype, which could not be ameliorated after applying istaroxime.

Author contributions

BG, RM, TdB, JWB and TvV designed the study. BG and RM conducted experiments and analyzed the data (RM: iPSC-CM differentiation, BG: electrophysiology). BG wrote the manuscript, TdB and TvV reviewed and edited the manuscript.

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Closing remarks

The development of patient-specific iPSC-CMs brought a tremendous potential to study diseases *in vitro*, screen drugs for tailored treatment and the possibility of erasing harmful mutations using genetic engineering. Until now, over 150 different iPSC-CM types of genetic cardiac syndromes have been reported^{1,2,3}. Most of these patient-specific iPSC-CMs recapitulate the mutation (genotype) of the patient and the effects of the mutation (phenotype). How the iPSC-CM translates to the disease *in vitro* has been evaluated in different ways. Studies report genetic markers, structural abnormalities, affected ion currents, action potential duration or field potentials representing a cellular ECG. One of the questions that remains is: what do we need for the scientific community to move forward?

Studies into disease modeling with iPSC-CMs highlight two major concerns: the lack of standardization in differentiation and the maturity of iPSC-CMs, which we have mentioned in this chapter. Cardiac differentiation protocols comprise of different methods, mostly resembling the stepwise differentiation in embryonic development, and variable spatiotemporal control over growth factors and media⁴. The result is time consuming, yielding different purities and cell numbers, all contributing to variability among iPSC-CMs. Genetic engineering introduces even more handling procedures: knocking in (or erasing) the mutation on an allele in a specific cell, then selecting the clones (low yield) that express the desired genotype and expanding the cells⁵. We observed large differences in the electrophysiological characteristics of DSP mutated iPSC-CMs when measured in different differentiation batches, illustrating the difficulty in standardization.

The second issue for moving forward is the maturity of iPSC-CMs, an aspect criticized from the time these models became available. As alluded to several times in this thesis, the structural organization and electrophysiology of iPSC-CMs is comparable to early embryonic cardiomyocytes impacting their translatability to the human situation. The depolarized resting membrane potential, the presence of the nodal funny current, the low levels of the inward rectifier potassium current and abnormal calcium machinery all contribute to the immaturity of iPSC-CMs⁶. When modeling diseases that have such a profound effect on electrophysiology as cardiomyopathies, the immaturity of iPSC-CMs is a major pitfall in modeling cardiac disease *in vitro*. The effects of the SERCA-activator istaroxime that we observed in the iPSC-CMs from a patient with a PLN mutation, were not in line with previous findings in iPSC-CMs and animal models. The lack of response might be explained by the immaturity of

iPSC-CM electrophysiology, although the differentiation capacity and protocol might skew the drug response as well.

In both studies we used either calcium-sensitive dyes or voltage- and calcium-sensitive dyes to assess the electrophysiological characteristics in patient-specific iPSC-CMs. These dyes respond fast to changes in membrane potential or intracellular calcium release, are non-invasive and allow higher-throughput measurements⁷. The choice for two separate dyes to assess action potential characteristics and calcium transients might not be convenient, as separate dyes might yield discrepancies in measurements. Additionally, dyes remain to be optimized for the use in iPSC-CMs⁸. Recently, an innovative tool was presented in which both voltage and calcium dynamics could be measured in one cardiomyocyte⁹. This increases the reliability of the measurements as it eliminates the variation possibly induced by administration of the separate dyes in different cells.

In both studies from this chapter we studied single patients with a mutation, one with ACM, the other with a pathogenic PLN Arg14del mutation. In order to draw conclusions from these studies several points need to be addressed to complete the studies. For the ACM study, the difficulty lies in the fact that we only studied one patient being the first and sole ACM patient that has been diagnosed with this mutation. We studied the patient's own iPSC-CMs and a knock-in of the patient mutation in a commercial line, thereby confirming the phenotype in an independent iPSC line. Due to the low efficiency of differentiation, we only measured the effect of one clone. More clones from the same individual will increase the sensitivity, although it would be more beneficial to include clones from more patients with the same mutation to fully assess the effect of the mutation *in vitro*¹⁰. For future PLN studies, we would also have to include iPSC-CM lines from multiple patients with a similar mutation in order to compare electrophysiology and include an isogenic control⁵. With these recommendations taken into account, patient-specific modeling using iPSC-CMs could become more specific and sensitive in order to truly recapitulate more aspects of the disease *in vitro*.

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Abstract

The human Ether-à-go-go Related Gene (hERG) encodes the pore forming subunit of the channel that conducts the rapid delayed rectifier potassium current I_{Kr} . I_{Kr} drives repolarization in the heart and when I_{Kr} is dysfunctional, cardiac repolarization delays, the QT interval on the electrocardiogram (ECG) prolongs and the risk of developing lethal arrhythmias such as Torsade de Pointes (TdP) increases. TdP risk is incorporated in drug safety screening for cardiotoxicity where hERG is the main target since the I_{Kr} channels appear highly sensitive to blockage. hERG block is also included as an important read-out in the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative which aims to combine *in vitro* and *in silico* experiments on induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) to screen for cardiotoxicity. However, the hERG channel has some unique features to consider for drug safety screening, which we will discuss in this study. The hERG channel consists of different isoforms, hERG1a and hERG1b, which individually influence the kinetics of the channel and the drug response in the human heart and in iPSC-CMs. hERG1b is often underappreciated in iPSC-CM studies, drug screening assays and *in silico* models, and the fact that its contribution might substantially differ between iPSC-CM and healthy but also diseased human heart, adds to this problem. In this study we show that the activation kinetics in iPSC-CMs resemble hERG1b kinetics using Cs^+ as a charge carrier. Not including hERG1b in drug safety testing might underestimate the actual role of hERG1b in repolarization and drug response, and might lead to inappropriate conclusions. We stress to focus more on including hERG1b in drug safety testing concerning I_{Kr} .

Introduction

The development of new drugs is a lengthy process. For a compound to gain approval to reach the market, it has to go through various stages of preclinical and clinical testing ranging from simple single cells to complex whole animal physiology. After that it needs to be approved by the Food and Drug Administration (FDA) in the US or the European Medicines Agency (EMA) in Europe. Only 1 out of 5000 compounds makes it through these stages, and bringing one compound to the market takes approximately 14 years and costs on average 1.5 billion US dollars¹.

Not only do few drugs enter the market, but they also suffer from a large attrition rate, often due to side effects. The range of side effects is broad, but cardiotoxicity accounts for about 45% of total post-approval drug withdrawal from the market². Drug-induced cardiac toxicity can impair the electrophysiology of the heart and cause prolongation of the QT interval on the electrocardiogram (ECG), which in turn can cause severe ventricular arrhythmias including lethal tachycardias such as Torsade de Pointes (TdP)³. Thorough preclinical safety assessment is performed to prevent cardiotoxicity from occurring during clinical trials or even after approval.

The golden standard for this type of preclinical drug screening consists of multiple levels that build up to increasing physiological complexity⁴. The first level consists of single cells that either originate from human cell lines and/or from animal cardiomyocytes (CM). Single cells represent the electrophysiological profile of the heart since they express all ion currents that are involved in action potential (AP) formation and propagation. Conventionally this is analyzed by patch clamp, which uses a few μm wide borosilicate glass pipettes to pinch a part of the cellular membrane and to clamp the cell at a certain voltage. After applying the drug, its effects on single ion currents can be accurately measured. A subsequent *in vitro* level of preclinical drug screens consists of testing the drug in animal ventricular myocardial wedges. These multicellular wedges do not only consist of cardiomyocytes, which influence their individual electrical activity in a 3D-fashion since their cytoplasmic environments are coupled by means of gap junctions, but also contain the supporting tissues (containing vasculature and fibroblasts) and allow to study AP propagation. The third level builds up in complexity and consists of testing the drug in whole animal hearts, which is subsequently followed by drug screening in whole animals and after that in clinical trials that also consists of different phases⁴.

At present, preclinical drug screening is cumbersome as it is time-consuming and not cost-effective. Most importantly, the system uses animals that physiologically differ from humans; so a drug that seems safe or unsafe in animals cannot automatically be considered safe or unsafe in humans⁵. Academia and industry have combined forces in order to reduce the amount of animals and humanize preclinical drug screening and created the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative⁶. CiPA resembles conventional preclinical drug screening in a number of ways; it uses single cells, focuses on I_{Kr} as a main target but differs since it is predominantly *in vitro* and completely human. CiPA does not rely on animal cardiomyocytes but on induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). iPSC-CMs are cardiomyocytes differentiated from reprogrammed human somatic cells such as fibroblasts, which are easy to yield. iPSC-CMs offer a plethora of possible *in vitro* purposes, such as disease modeling and drug screening, but also regenerative medicine⁷. CiPA consists of multiple phases just as conventional drug screening. In phase 1, drugs are tested in human cell lines for their effect on ectopically expressed individual ion currents. In the second phase, the findings are used in combination with an *in silico* model of the human ventricular action potential to predict the changes on action potential duration (APD). In the third phase the drug is tested in iPSC-CMs, followed by clinical evaluation in the fourth phase.

The CM is an excitable cell and therefore expresses a different number of various ion channels that might be influenced by drugs. However, one specific ion current is particularly affected by drugs: the rapid part of the delayed rectifier potassium current (I_{Kr}), conducted by the human Ether-à-go-go Related Gene (hERG) channel which is involved in phase 3 of the cardiac action potential where it facilitates repolarization⁸. When I_{Kr} is dysfunctional either due to a mutation or drug-induced, the CM cannot properly repolarize which prolongs the APD and this is visible on the human ECG as a prolonged QT interval. Prolonged QT intervals may be harmless, but can progress into arrhythmias such as TdP. The risk for TdP has been included in guidelines since 2005 for preclinical (S7B) and clinical (E14) screening, which makes hERG therefore a hallmark of drug safety⁹.

In this study we focus on the different characteristics of the hERG channel and how they influence drug safety screening assays. With the rapid development of CiPA it is key to understand how the characteristics of this channel may impact safety testing. We will discuss the hERG channel characteristics following the work flow as presented in the CiPA initiative; 1) the structure and kinetics of the hERG channel, 2) the *in silico* models of hERG channels and 3) hERG channels in iPSC-CMs. Finally,

different electrophysiological methods to record and characterize I_{Kr} are discussed. We also present new work on classifying I_{Kr} , where we employ an alternative patch clamp approach with Cs⁺ to unravel the kinetics of the Cs⁺-carried hERG current in iPSC-CMs.

Characteristics of hERG

The electrical activation of the heart is initiated as an AP in the sinoatrial node which spreads through the conduction system to CM in the working myocardium where it causes the heart to contract. The AP is the result of an orchestrated activation of voltage-sensitive ion channels. First, sodium channels open in response to a depolarizing stimulus, allowing a rapid influx of sodium ions, resulting in the upstroke of the AP (phase 0). The transient outward potassium current (I_{to}) then facilitates rapid but limited repolarization (phase 1); because repolarization is counterbalanced by the opening of calcium channels that allow the influx of calcium ions, creating the plateau phase (phase 2). The action potential is terminated by final repolarization driven by I_{Kr} and I_{Ks} (phase 3), which also induces the closing of the calcium channels, after which the resting membrane potential is maintained by the inward rectifier potassium current (I_{K1} , phase 4) (Figure 1).

Figure 1: The cardiac ventricular action potential.

Illustration of the action potential of a human adult ventricular cardiomyocyte indicating the 5 phases of the action potential and the dominant ion currents, based on a simulation done using the ten Tusscher 2006 model.

Channel structure

The hERG channel consists of four monomers that each contain six helices (S) that span the membrane and each helix has its own special function. The voltage sensing domain is formed by S1-S4, a voltage linker by S4-S5 and the pore is formed by

S5-S6¹⁰. The pore is selective for potassium ions, which is commonly referred to as a selectivity filter. Traveling to the inside of the cell, we observe that the pore contains several cytoplasmic domains. On the C-terminal end, the channel contains a Cyclic Nucleotide Binding Homology Domain (CNBD) and on the N-terminal end an Ether A Go go (EAG) domain composed of a Per-Arnt-Sim (PAS) domain and a PAS-cap domain. A deconstructed view of the hERG channel is visualized in Figure 2. The cytoplasmic domains are inherent to the gating kinetics of the channel. The channel produces small outward currents during depolarization and large currents during repolarization. During the course of the cardiac AP, depolarization activates the channel slowly, but subsequently it inactivates very quickly, only for it to recover from inactivation and reopen during repolarization. The reopened channels then deactivate slowly and thus slowly close, allowing the potassium flow to fulfil its role as repolarizing current¹¹. The pore structure together with the gating mechanism is visualized in Figure 2.

Recently, the structure of the hERG channel has been examined in more detail using cryo-electron microscopy¹². The channel is suggested to be open with the voltage sensor at depolarized voltages where the S5-S6 are influenced by a conformational change in S4. However, the classical view that S4-S5 acts as a voltage lever is challenged, since recent studies suggest that electrostatic interactions between the PAS-cap and the transmembrane domains contribute to opening of the channel^{13, 14}. It is furthermore suggested that the pore contains pockets with hydrophobic amino acids that have a high affinity for drug binding which might explain the susceptibility of the hERG channel for so many drugs. Next to that, the open cavity in the pore is very small and electronegative, which adds to the drug binding properties¹². This recent work offers new insight into the channel structure, although the inactivation mechanism is yet to be elucidated.

If channels lack the EAG domain they inactivate slower, recover faster from inactivation and do not reach steady state inactivation as channels that do possess an EAG domain¹⁵. The EAG and CNBH domains interact with each other to promote slow deactivation to maximize the amplitude of the current during repolarization in phase 3 of the action potential while returning to its quiescence at phase 4 of the action potential¹⁶.

Figure 2: The structure and gating kinetics of the hERG channel.

(A) The hERG channel consists of six transmembrane helices with voltage-sensing domain in S4, indicated by the positive charges along the vertical axis. The channel contains a PAS-containing EAG domain at the N-terminus and a CNBD domain at the C-terminus. (B) The hERG channel is closed and opens upon depolarization. The PAS- and CNBD domains move towards the cell membrane, after which the pore opens. It inactivates very quickly and then recovers from inactivation during repolarization. The hydrophobic pockets in the pore increase the affinity for drugs. IC=intracellular; EC=extracellular.

hERG isoforms

The channel is formed as a tetramer, containing four subunits that differ structurally and functionally. Three isoforms of human hERG have thus far been reported: hERG1a¹⁷, hERG1b^{18, 19} and hERGuso²⁰. hERGuso does not form functional channels by itself, but when present in excess it can modify the kinetics in hERG1a/hERGuso channels. Like hERGuso, hERG1b differs from hERG1a due to alternative transcription, and has a truncated N-terminus thereby lacking the EAG domain. As explained before, the EAG domain ensures fast inactivation, so in absence of this domain inactivation is slow,

inactivation is incomplete and the channel recovers faster from inactivation¹⁵. Next to that, hERG1b activates faster and deactivates faster²¹. More recently, experimental data together with kinetic modeling suggests that hERG1b is the driving force for recovery from inactivation, which is in accordance with previous studies¹⁴.

hERG channels in the cardiomyocyte do not comprise solely of hERG1a or hERG1b isoforms but prefer to form heterotetramers²². The ratio of hERG1a and hERG1b in heterotetramers in the human heart is not yet fully understood, but has predicted to be a ratio of 1a:1b of 2:2 or 3:1²². When hERG1a is mutated and folded in homomers there is no decrease in I_{Kr} , but when hERG1a is mutated and folded in heteromers with hERG1b, negative gating and trafficking is observed²³. hERG1b is an important driver for repolarization and is essential for cardiomyocyte function. Specific hERG1b mutations have been associated with intrauterine fetal death^{24, 25}. Human failing hearts had substantial downregulation of I_{Kr} that was accompanied by an increase in the hERG1b isoform abundance compared to control hearts²⁶.

Next to different gating kinetics between the two isoforms, drug response also seems to differ between hERG1a and hERG1b. In HEK293 cells overexpressing 1a, and 1a/1b, 1a channels were blocked more rapidly by the hERG specific blocker E-4031 than 1a/1b channels²⁷. The same held true for dofetilide²⁸. Also the I_f blocker ivabradine affects isoforms differently; the IC_{50} for hERG1b was comparable to I_f channels, which was a two-fold lower than the IC_{50} for hERG1a²⁹. The difference in drug response might be due to the different kinetics, since 1a/1b channels spend more time in the open state and are therefore more prone to block²⁸. 1b is also more influenced by acidosis than 1a or 1a/1b channels³⁰. The different kinetics and drug response of the isoforms are not only vital for hERG function, but might also have consequences for the outcomes of drug screening assays.

hERG in iPSC-CMs

hERG isoforms in iPSC-CMs

The development of iPSC-CMs has given *in vitro* methods a tremendous boost. The ability to produce CM from somatic cells eliminates multiple constraints for cardiac research as iPSC-CMs are easy to yield and represent a human physiological model of cardiomyocytes to a certain level. Although iPSC-CMs present multiple issues in terms of immature morphology and electrophysiology³¹, it is continuously used as a model to study cardiac disease. iPSC-CMs have been described to contain hERG current, though it has a small current density³². Many different studies report

hERG gene and protein expression levels for iPSC-CMs, although this is often on hERG1a and therefore does not provide a complete picture. Only some studies have analyzed the role of hERG1b in iPSC-CMs. Reducing hERG1b expression to $\pm 50\%$ by shRNA in iPSC-CMs prolonged action potential duration, increased variability between action potentials and caused early afterdepolarizations³³. Prolonged culture increases hERG1b expression in hESC-CMs^{34, 35, 36} and influences sensitivity to E-4031³⁵. Interestingly, hERG1b is often the most abundant isoform in neonatal mice hearts, while hERG1a is the most abundant isoform in adult mice hearts³⁷. At present there is no data on the balance between hERG1a and hERG1b in human cardiac development, but given the expression of hERG1b and its influence on repolarization it is likely that also in the human heart the amount of hERG1b is not fixed. Variations in hERG1a/hERG1b balance may therefore influence I_{Kr} and drug response as in iPSC-CMs.

LQTS Type II iPSC-CMs

I_{Kr} dysfunction, either drug-induced or congenital, can give rise to Long QT Syndrome (LQTS) type II. Patients that suffer from LQTS type II have a higher risk on developing arrhythmias, which can lead to ventricular arrhythmia (such as TdP) and ultimately sudden cardiac death (SCD). In LQTS type II the Kv11.1 protein, which constitutes the I_{Kr} channel, is not functional, leading to an improper hERG channel function. Mutations of LQTS type II can be classified into four categories: mutations affecting the transcription and translation of Kv11.1 alpha subunits (class 1), disrupted trafficking of the protein to the membrane due to misfolding or aberrant quality control in the Endoplasmic Reticulum (ER) (class 2)^{38, 39}, or aberrant gating / permeation of the channel (class 3 and 4 respectively)⁴⁰. As mentioned before, the development of iPSC-CM technology allows the study of patient specific material *in vitro*. To study the mechanisms that underlie LQTS, fibroblasts can be taken from the patient and reprogrammed to cardiomyocytes harboring the LQTS mutation. Patient specific iPSC-CMs for LQTS are generally used to understand how certain mutations cause the disease, to uncover the mechanism of disease or to study the response to newly developed therapies⁴¹. Novel genetic engineering techniques such as CRISPR/Cas9 take therapy a step further by giving the possibility of reverting the disease phenotype by correcting the mutation⁴².

The first patient specific LQTS type II iPSC-CMs were developed by Itzhaki *et al.* and after that, many different iPSC-CMs have been created with a plethora of LQTS Type II causing mutations⁴³. Studies into patient specific iPSC-CMs for LQTS type II follow a common pattern. The iPSC-CMs are first genotyped whether they harbor the mutation, stained for cardiomyocyte specific markers and subjected to a series of

electrophysiological measurements to conclude whether the iPSC-CMs recapitulate the arrhythmogenicity *in vitro*⁴¹. Single cells can be analyzed for their APD and LQTS type II specific APD prolongation. Monolayers of iPSC-CMs allow analysis by Multi Electrode Arrays (MEA) in which the field potential duration (FPD) is determined that acts as a cellular ECG⁴⁴. iPSC-CMs can then be subjected to drugs to evaluate the arrhythmogenic or relieving effects.

iPSC-CMs have enabled to unravel several aspects of the LQTS Type II disease mechanism, such as aberrant splicing⁴⁵ and the role of calcium in LQTS. LQTS supposedly acts on cytosolic calcium handling to promote the arrhythmic phenotype⁴⁶. Aberrant calcium handling has also been identified in other lines^{40, 47} and implemented in the *in silico* Paci iPSC-CM model to study calcium handling abnormalities in more detail⁴⁸. Most studies focus on relieving the arrhythmogenic phenotype in LQTS iPSC-CMs. Correction of defective trafficking of Kv11.1 was established by small molecule ALNN⁴⁹. The clinically available drug lumacaftor decreased the accumulation in iPSC-CMs harboring a class II mutation and restored trafficking of the channel to the membrane⁵⁰. This compound also normalized irregular calcium transient amplitudes and timing. Inhibiting the GTP-binding protein REM2, which is a family member of the cell cycle promoting Ras, by genome editing relieved the arrhythmogenic phenotype in a different LQTS2 iPSC-CM line⁴⁷. In addition, several genetic variants of LQTS type II were reversed by CRISPR/Cas9⁵¹. A large-scale analysis of clinically reported LQTS Type II mutations showed that the effects on trafficking of most, but not all, mutations can be corrected *in vitro* by lowering temperature (from 37°C to 27°C) and/or addition of the hERG blocker E-4031⁵². Mutations in cytoplasmic domains could be corrected, while pore mutations could not. This was further aggravated if the channel was composed as a homomer, consisting of subunits of a single isoform.

Mathematical models of I_{Kr}

The first mathematical model of an excitable cell was created by Hodgkin and Huxley who described the biophysical characteristics of action potential propagation in the squid giant axon⁵³. Later, Noble applied the same principles to CM, as their cell membrane can be also be considered as an electrical system with a capacitance and ion channels acting as conductances⁵⁴. Modeling ion channels or the AP propagation in CM allows the reproduction of wet experiments and the expansion of knowledge to gain insight into channel structure, function or drug binding.

The kinetics of the hERG current can be modeled according to the Hodgkin-Huxley (HH) formulation. However, HH formulations model activation and inactivation as independent gating processes. Since hERG displays unusual kinetics regarding activation and inactivation, applying the HH formulation to model hERG may not capture the full kinetics of activation, as it may yield unreliable time constants for activation. HH formulations are therefore generally not preferred in modeling hERG¹¹. Instead, hERG can also be modeled according to Markov formulations which may represent a more physiologically relevant set of equations. Markov models consist of different states that are either open, closed or inactivated and transitions with various rates that relate to the different gating processes *i.e.* activation, inactivation, recovery from inactivation and deactivation¹¹. Most models contain three closed states, one inactivated state and one open state. The presence of three closed states allow accurate description of the multiple time constants observed in activation. The configuration of the different states can be linear or branched, and the transition rates can differ, which yields a plethora of possible Markov configurations.

Several general hERG models are now available. Wang *et al.* created a linear five state Markov model with three closed states and included a voltage-independent step of activation⁵⁵. This was further expanded by Clancy & Rudy who created a branched Markov model which was based on the Wang model, but also included a step from the third closed state to the inactive state⁵⁶. Manzari *et al.* created a Markov model also based on the Wang model, but with a different transition rate from closed to open than from closed to inactive⁵⁷. An overview of the different Markov models is given in Figure 3. Markov models can be used to complement and interpret experiments. Piper combined experiments in *Xenopus laevis* oocytes with Markov modeling to elucidate dependence of activation and inactivation gating⁵⁸. AP clamp and dynamic clamp were used to optimize the Luo-Rudy formulation of guinea pig I_{Kr} that reproduced experimental findings more accurately. One of the changes was the inclusion of the voltage dependence of inactivation⁵⁹.

Markov formulations of hERG can also be incorporated into action potential models to examine the effects on APD or to simulate drug response. Fink and colleagues recreated the ten Tusscher AP model with a five state Markov model instead of the original HH formulation⁶⁰. Mazhari *et al.* incorporated their new Markov formulation of I_{Kr} into a canine midmyocardial action potential model to unravel the interaction between hERG and KCNE2, which can give rise to acquired LQTS⁵⁷. Romero *et al.* combined a specific LQTS Type II mutation with the Fink Markov formulation of I_{Kr} into the O'Hara-Rudy ventricular action potential model to screen for I_{Kr} blockers⁶¹.

A **B** **Figure 3: Markov models of the hERG channel.**

Examples of Markov model topologies. C1-C3=closed state, O=open state, I=inactivated state.

(A) Linear Markov model according to Wang *et al.* **(B)** Branched Markov model according to Clancy & Rudy and Manzari *et al.*

As knowledge about the hERG channel grows and experiments unravel hERG kinetics in more detail, Markov models are being refined as well. The hERG channel behaves differently at room temperature than at physiological temperature, which influences the kinetics and drug response. The temperature dependence in models is often implemented using the temperature coefficient Q_{10} , which unfortunately does not apply to hERG⁶². Recently, the temperature dependence of hERG was incorporated into a Markov model, for the model to respond properly to temperature changes⁶³. This Markov formulation has been included in the O'Hara-Rudy action potential model⁶⁴ and combined with multichannel pharmacology. The model has gained attention from the CiPA society and the model is further developed with CiPA drugs to predict TdP risk⁶⁵. Since temperature also impacts drug binding, incorporating the temperature sensitivity of I_{Kr} might also promote proarrhythmia during *in silico* assessment⁶⁶.

One of the prominent limitations of mathematical modeling studies is the variability in experimental data used in construction of the models. The sources for experimental differences between laboratories can be explained by the use of different experimental techniques such as voltage protocols, the use of different cell types and lines and experimental conditions such as solutions. Even if these parameters are standardized, the biological variability between cells remain. Another challenge lies in drug binding, as some drugs bind with different kinetics, may or may not be trapped in the channel once it is closed and impede hERG activation differently as a

result^{67, 68}. Altogether, technical, experimental and mechanistic gating details need to be taken into account in order to develop a properly predictive model.

How to measure I_{Kr} using patch clamp and specifically in iPSC-CMs

Measuring ion currents in a single cell requires precise techniques and therefore manual voltage (patch) clamp remains the golden standard to characterize I_{Kr} . The voltages that are applied to the cell elicit different modes of I_{Kr} activation and offers insight into the channel kinetics. Activating I_{Kr} yields current densities and thus conductance, as well as the voltage at which half of the current is activated which is referred to as the half-maximal activation ($V_{1/2}$).

The voltage steps for different types of activation needs to be applied separately to the cell, leaving the cell prone to changes during the course of the experiment in *e.g.* seal resistance and increasing the variability between measurements. Recently, Beattie *et al.* showed that short (8 second) sinusoidal wave protocols can be used to rapidly extract enough information from a patch clamp experiment to construct a cell-specific hERG model based on HH formulations. The value of making cell-specific models was underlined by the finding that cell-specific models were better able to predict hERG currents in response to AP clamp stimuli than models based on the average data of multiple cells. The cell-specific HH models also gave better predictions than Markov models from literature^{55, 57, 69, 70, 71}. Also, the predictive quality of cell-specific models was influenced by the quality of the experimental data used to fit the model; larger leak currents implied less accurate prediction of experimental data^{72, 73}. Accurately capturing the biological variability between cells is not the only focus of ongoing research. In the past few years, the development of automated patch clamp systems has taken a big leap. Automated patch clamp devices can patch multiple cells at the same time, thereby saving time and labor intensity. One of the main advantages of automated patch clamp is the increased throughput, since it is possible to simultaneously patch cells ranging from 4 to 384 cells at the same time^{74, 75}. Furthermore, automated patch clamp devices have been combined with automated dynamic clamping⁷⁶. These developments could boost preclinical drug screening initiatives such as CiPA. The use of iPSC-CMs in CiPA could pose additional challenges in characterizing I_{Kr} , since the small size of iPSC-CMs (cell membrane capacitance 20-50 pF⁷⁷) means that even if they express current densities that are equal to those of adult cardiomyocytes (typically ~0.5 - 1 pA/pF), absolute current magnitude is expected to be very small (between 10 - 30 pA). This is especially troublesome if the experiment requires application of drugs, which takes time during

which experimental conditions may vary to such an extent that calculating a drug-sensitive current component is no longer feasible.

Not only does the machine on which the measurements are performed or which protocol you apply influence the way I_{Kr} is measured, also the solutions in which the cell is embedded matters. hERG is a potassium channel and in patch clamp the potassium current is measured. However, cardiomyocytes contain other potassium channels (*i.e.* I_{K1} , I_{to} , I_{Ks} , I_{KACh}) as well, so it is likely that the current that is measured during patch clamp experiments is not solely I_{Kr} , but is also contaminated by other currents⁷⁸. This poses difficulties for drug safety screening, as drug effects might also be influenced by the solutions surrounding the cardiomyocyte possibly leading to false positive or negative results.

However, an alternative for potassium exists that isolates I_{Kr} and prevents contamination by other potassium carried currents: the use of equimolar Cs⁺ containing solutions. Cesium ions are known to block other potassium currents such as I_{K1} and I_{Ks} , and have been observed to be conducted specifically by I_{Kr} channels⁷⁸. Since the pore of the hERG channel is narrow, the selectivity filter only passes a number of ions such as cesium. Equimolar Cs⁺ solutions can be used in manual patch clamp experiments to measure the Cs⁺-carried I_{Kr} , giving a more selective measure of I_{Kr} than conventional experiments using K⁺ as a charge carrier. Additionally, when measured using Cs⁺ ions, I_{Kr} current amplitude is much larger, up to 10 times as large. Not many studies have employed the equimolar Cs⁺ approach so far, and have not considered hERG1b. If I_{Kr} can be measured reliably using Cs⁺, the approach may also be particularly useful to distinguish the contribution of different hERG isoforms. Patch clamp using Cs⁺ has been successfully applied to human cell lines, dog⁷⁹, rabbit⁷⁸ and rat cardiomyocytes⁸⁰, but not yet in iPSC-CMs. Therefore, we applied the equimolar Cs⁺ based patch clamp approach in the human cell line HEK293T transfected with the different isoforms and determined Cs⁺ current density and $V_{1/2}$. In addition, we determined the Cs⁺-carried I_{Kr} current density and $V_{1/2}$ in iPSC-CMs.

Methods

Cell culture

iPSC-CMs were kindly provided by Prof. Zimmermann and Dr. Tiburcy from the University Medical Center of Goettingen, Germany. iPSC-CMs were cultured on Matrigel coated coverslips in RPMI with 2% B27 serum replacement (ThermoFischer Scientific), 1% Non-Essential Amino Acids (ThermoFischer Scientific), 1% sodium

pyruvate (ThermoFischer Scientific), 2 mM ascorbic acid (Sigma-Aldrich) and 1% penicillin/streptomycin (ThermoFischer Scientific) (adapted from ⁸¹). HEK-hERG ⁸ and HEK293T cells were cultured in high-glucose DMEM supplemented with 10% Fetal Calf Serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

Transfection

hERG1a and hERG1b constructs were kindly provided by prof. dr. Thomas Jespersen from the University of Copenhagen. hERG1a and hERG1b were cloned into pXOOM vectors. HEK293T cells were transfected using Lipofectamine (4 µl in 100 µl DMEM) mixed together with 0.25 µl GFP (1 µg/µl) and 0.75 µl pcDNA of either hERG1a or hERG1b (1 µg/µl) in 100 µl DMEM. The two solutions were mixed and after 20 minutes added to a 10 cm dish with a 40-50% confluent cell layer. After 20-24 hours, cells were split and seeded on 10 mm cover slips coated with poly-L-lysine for patch clamping.

Fluorescent immunohistochemistry

Poly-L-lysine coated coverslips with transfected HEK293T cells were washed once with DMEM, fixed using Methanol at -20°C, and washed three times using PBS to remove any methanol. Coverslips were stored in PBS at 4°C until used for immunocytochemistry. Before applying antibodies, cells were permeabilized with 0.2% Triton X-100/PBS. Non-specific binding of antibodies was blocked with 2% bovine serum albumin (BSA) for 45' at RT. Incubation with primary antibodies was performed overnight in PBS/10% normal goat serum (NGS). Immunolabeling was performed using Texas Red (TR)- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. All incubation steps were performed at room temperature and in between incubation steps cells were washed with PBS. Finally, sections were mounted in Vectashield, coverslips were placed, sealed with nail polish and examined with a Nikon Optiphot-2 light microscope equipped for epifluorescence. hERG1a and hERG1b were stained using anti-hKv11.1 from Alomone (rabbit, 1:150). DAPI was used as a marker of the nucleus.

Protein isolation and western blot

Protein lysates were made using 490 µl Ripa buffer, 5 µl PMSF 200 mM and 5 µl Aprotinin per T25 flask. The concentration was determined using a BCA assay. The protein lysate was diluted with sample buffer to a concentration of 25 µg/ 20 µl, and stored at -20°C. Western blots were performed using the same anti-hERG antibody from Alomone (1:7000) as used for immunohistochemistry.

Electrophysiology

Cs⁺-carried I_{Kr} measurements were carried out at room temperature on a HEKA EPC-10 Double Plus Amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by Patchmaster 2.73. The extracellular solution contained (in mM): CsCl 135, MgCl₂ 1, glucose 10, HEPES 10, nifedipine 0.01, adjusted to pH 7.2 with CsOH. The pipette solution contained (in mM): CsCl 135, MgCl₂ 1, glucose 10, HEPES 10, adjusted to pH 7.2 with CsOH. The pipette resistance was between 2 and 4 M Ω . Series resistance was compensated to 70% with a time constant of 100 ms.

Cs⁺-carried I_{Kr} was recorded using a holding potential of -80 mV, then I_{Kr} was activated with 3s test pulses ranging from -70 mV to 70 mV with 10 mV increments. After the test pulse the cell was clamped back to the holding potential of -80 mV for 2 s in which tail currents were elicited and the channel slowly closed. The interval between pulses was 10 s.

Data analysis

Tail currents were measured and plotted versus test potential in an I-V diagram. I-V diagrams were fitted with a single Boltzmann function to calculate the half-maximum activation voltage ($V_{1/2}$) and the slope factor (steepness of the voltage dependence of gating) of activation. All values are presented as mean $\pm$ SEM.

Results

Stable hERG isoform expression in HEK293T

HEK cells were transfected with either hERG1a or hERG1b. The expression was first analyzed by western blot, where non-transfected 293T were used as a negative control and the stable cell line HEK-hERG as a positive control (Figure 4). HEK-hERG is usually characterized by two bands: a mature, which is fully glycosylated, and an immature, which is only glycosylated at the core. hERG1a is visible at 135 kDa as the immature form. hERG1b is visible at 85 kDa, which is suspected to be an immature band as well. Subsequently the cells were immuno-labelled for hERG, with DAPI serving as an intracellular marker to delineate the nucleus. All constructs are mostly localized at the plasma membrane, which indicates appropriate expression and trafficking of the overexpressed hERG channels (Figure 4).

Figure 4: Protein levels of hERG1a/1b and immunohistochemistry.

(A) Protein expression of hERG1a (135 kDa) and hERG1b (85 kDa). Non-transfected HEK cells and HEK-hERG cells served as a control. (B) Transfected HEK293T cells with either hERG1a or hERG1b were stained for hERG and DAPI, showing clear signal at the cell membrane. Non-transfected HEK cells served as a control.

Activation kinetics of Cs⁺-carried I_{kr} in HEK293T

The activation of hERG1a or hERG1b was determined in HEK cells by using whole cell patch clamp. For that the cells were clamped at a holding potential of -80 mV. After an initial pulse at the holding potential for 750 ms, the currents were measured from -70 mV to 70 mV in 10 mV depolarizing steps of 3 s. After that tail currents were evoked by jumping back to -80 mV for 2 s, as can be observed in Figure 5A and B.

The average tail current density after a step to 20 mV, when the tail current was fully activated, was -532.5 ± 167.0 pA/pF for 1a and -22.0 ± 4.2 for 1b. The amplitudes of the tail current were normalized to the largest tail current and plotted against the depolarizing voltages. The data were fitted to a Boltzmann equation (Figure 5D+E), after which the $V_{1/2}$ could be derived. The $V_{1/2}$ for hERG1a was -41.1 ± 1.6 mV (n=6), while $V_{1/2}$ for hERG1b was -29.8 ± 8.4 mV (n=6) (Figure 6).

Figure 5: Cs⁺-carried I_{Kr} activation kinetics in HEK293T cells and iPSC-CMs.

Representative traces for Cs⁺-carried I_{Kr} activation recorded in HEK293T expressing hERG1a or hERG1b and iPSC-CMs (upper panel). Normalized tail current plotted against test potential and subsequently fitted with a Boltzmann equation for hERG1a, hERG1b and iPSC-CMs (lower panel).

Activation kinetics of Cs⁺-carried I_{Kr} in iPSC-CMs

Cs⁺-carried I_{Kr} was recorded in iPSC-CMs using whole cell-patch clamp according to the same protocol as was applied in the HEK293T cells, as can be observed in Figure 5C. Fitting the normalized tail currents to the test voltages and fitting the data to a Boltzmann equation (Figure 5F) yielded a $V_{1/2}$ in iPSC-CMs of -29.76 ± 3.31 mV (n=7)

(Figure 6). The average tail current density at -80 mV was -6.4 ± 1.2 pA/pF after the 0 mV step (n=6), -6.6 ± 1.6 pA/pF after the 20 mV step (n=6) and -7.1 ± 1.5 pA/pF after the 60 mV step (n=6).

Figure 6: Voltage at half-maximal activation ($V_{1/2}$) of 293T cells and iPSC-CMs. $V_{1/2}$ for hERG1a and hERG1b in 293T cells and iPSC-CMs, determined from the activation kinetics.

Discussion & Conclusion

In this study we focused on hERG1a and hERG1b, which are two transcripts of the hERG gene that give rise to different protein subunits that can form tetramers and constitute the ion channel conducting I_{Kr} . The ratio of those isoforms affects the different modes of activation of the I_{Kr} current, but also influences the response of drugs that interact with the channel. In addition, the expression of hERG1a and hERG1b might differ between adult human cardiomyocytes and more immature CMs (e.g. embryonic stem cell-, or iPSC derived CM). The latter ones are thought to become an important tool in the CiPA initiative: a relatively new platform for *in vitro* safety testing of drugs. In this study, we reviewed the current body of literature on the two isoforms, and aimed to elucidate the hERG1a/hERG1b ratio in a specific line of iPSC-CMs by determining the half-maximal voltage at which the current is activated ($V_{1/2}$). The experiments were performed using equimolar Cs^+ solutions, which enlarged the Cs^+ -carried I_{Kr} current density.

The $V_{1/2}$ we measured for the iPSC-CMs was close to -30 mV, which is indistinguishable from the $V_{1/2}$ determined in HEK293T cells expressing hERG1b alone, and ~10 mV less negative than the $V_{1/2}$ we found in HEK293T cells solely expressing hERG1a. Moreover, the $V_{1/2}$ reported for rabbit and neonatal rat ventricular myocytes (Table 1) or heteromeric hERG1a/1b channels in HEK293 cells^{27, 82} is very similar to that found in iPSC-CMs. Taken together, our data indicates that hERG1b is functionally present in iPSC-CMs and influences the kinetics.

The maximal tail current density we measured for Cs⁺-carried I_{Kr} in iPSC-CMs was 6.6 ± 1.6 pA/pF, which is about six times larger than I_{Kr} reported in iPSC-CMs using potassium based solutions^{32, 33, 41, 49}. The advantage of using Cs⁺ is that it will result in a larger and hERG channel specific I_{Kr} current, as other K⁺ channels will not conduct Cs⁺ ions⁷⁸. Cs⁺ can increase the I_{Kr} current density up to a 4-fold in neonatal rat cardiomyocytes⁸³. A benefit of the hERG specificity of Cs⁺ carried current that should not be missed is that it is not necessary to block the ion current recorded with a known gold standard I_{Kr} blocker (e.g. E-4031 or dofetilide) in order to determine the identity of the current. This is particularly relevant when testing new, unknown drugs as there is no risk of undetected (cooperative or competitive) interactions between the drug being tested and the gold standard blocker. As such, the use of Cs⁺ might be well-suited for studies aiming to elucidate the characteristics of hERG1a/hERG1b channels, and the isoform-specific effects of drugs.

The existence of hERG1a and 1b isoforms in iPSC-CM and their mutual expression levels have not been addressed in most studies using these rather immature cardiomyocytes, while the isoforms could be of utmost importance for drug screening. The relevance of iPSC-CMs as a model for drug screening not only depends on the molecular expression of hERG1a and 1b, but also on their generalized ability to recapitulate the electrical characteristics of the human adult ventricular cardiomyocyte. iPSC-CMs are known for their immaturity and current strategies aim to standardize differentiation, improve maturity status and decrease variation. Regarding the electrophysiological phenotype, most often attention is focused on the unstable and depolarized resting membrane potential (caused by a disturbed balance between I_{K1} and I_f) and the aberrant calcium handling. However, the differentiation and maturation strategies need to be further developed to increase the reliability of iPSC-CMs as a cellular model for CiPA⁸⁴, and ensuring a representative hERG1a/hERG1b ratio in iPSC-CM is strongly advised.

Mathematical modeling of hERG is a key aspect of CiPA that constantly evolves. The recent inclusion of hERG1a/hERG1b isoforms have improved the performance of action potential models^{14, 27}. hERG isoform specific models could be combined with experimental data from drug assays obtained in high-throughput patch clamp experiments done using cell lines expressing hERG1a, hERG1b or both. If this approach could be combined with voltage clamp stimuli that allow very fast determination of all relevant hERG kinetics in a few seconds, cell-specific models can be made that can capture the hERG current characteristics both in absence and presence of drugs. In essence, cell-specific hERG models can be used as an information-packed description of the experimental data that can be used in subsequent action potential modeling to predict the effects of drugs on CM excitability. Validation is also key for *in silico* models, as they need to be translational across different data set obtained at different institutions^{65, 85}.

Data from *in vitro* and *in silico* studies show the added value of including hERG1a/hERG1b isoforms into hERG studies, while the importance for drug screening might have been underestimated thus far⁶. To conclude, we propose that more focus should be given towards hERG isoforms in iPSC-CMs as they importantly influence drug response and thus need to be implemented in *in vitro* drug screening approaches.

Table 1: Characteristics of I_{Kr} in human cell lines expressing hERG1a, hERG1b, hERG1a/hERG1b, and iPSC-CMs

Isoform	Type of protocol	Temperature (°C)	Ion used (K/Cs)	$V_{1/2}$ (mV)	Slope	Current density in pA/pF	Cell type
1a	Holding potential (HP) -80 mV	Room temperature (RT)	Cs	-41.2 ± 2.0	6.4 ± 0.4	52.5 ± 3.3 at -80 mV after depolarizing step to 20 mV	HEK293 ⁷⁸
	-70 to 70 mV in 10 mV increments						
	Step to -80 mV						
	HP -80 mV	34 ± 2	K	-26.8 ± 1.3	6.9 ± 0.2	/	HEK293 ²⁷
	Depolarization to 20 mV						
	Tail at -50 mV						
	HP -80 mV	RT	K	-2.8 ± 1.6	8.6 ± 0.2	At the end of a 1s depolarizing pulse to 0 mV	CHO ²¹
	-100 to 60 mV in 10 mV increments						
	Tail at -60 mV					15.2 ± 2.2	
	HP -80 mV	37	K	-20.08 ± 0.78	7.89 ± 0.79	Maximal current at -37.60 ± 2.31 mV (no current density mentioned)	CHO ⁸⁶
	-80 to 100 mV in 10 mV increments						
	Tail at -40 mV						
	HP -80 mV	RT	K	/	/	Peak potential at -39.6 ± 1.7 mV with peak amplitude of 0.39 ± 0.01	HEK293 ⁸⁷
	Clamp at 40 mV						
	Ramp to -80 mV						
	HP -80 mV	20-22	K	/	/	67.1 ± 10.2	HEK293 ⁸⁰
	-60 to 60 mV						
	Tail at -50 mV						
	HP -80 mV	RT	Cs	-41.1 ± 1.6	/	/	HEK293T (this study)
	-70 to 70 mV in 10 mV increments						
Tail at -80 mV							

Table 1: Continued

Isoform	Type of protocol	Temperature (°C)	Ion used (K/Cs ⁺)	V _{1/2} (mV)	Slope	Current density in pA/pF	Cell type
1b	HP -80 mV	RT	K	-12.4 ± 1.3	9.1 ± 0.3	At the end of a 1 s depolarizing pulse to 0 mV	CHO ²¹
	-100 to 60 mV in 10 mV increments						
	Tail at -60 mV					23.9 ± 3.1	
	HP -80 mV	RT	K	/	/	Peak potential at 1.9 ± 1.9 mV with peak amplitude of 0.37 ± 0.01	HEK293 ⁸⁷
	Clamp at 40 mV						
1a/1b	Ramp to -80 mV						
	HP -80 mV	RT	Cs	-29.8 ± 8.4	/	/	HEK293T (this study)
	-70 to 70 mV in 10 mV increments						
	Tail at -80 mV						
	HP -80 mV	34 ± 2	K	-28.6 ± 1.0	6.2 ± 0.1	/	HEK293 ²⁷
	Depolarizing steps to 20 mV						
	Tail at -50 mV						
	HP -80 mV	RT	K	-13.8 ± 1.1	8.4 ± 0.2	At the end of a 1 s depolarizing pulse to 0 mV 48.6 ± 9.1	CHO ²¹
	-100 to 60 mV in 10 mV increments						
	Tail at -60 mV						
	HP -80 mV	37	K	-27.0 ± 0.4	7.4 ± 0.4	Maximal current at -22.3 ± 1.0 during action potential	HEK293 ⁸²
	Jump to 20 mV						
	HP -80 mV	RT	K	/	/	Peak potential at -19.3 ± 2.9 mV with peak amplitude of 0.35 ± 0.01	HEK293 ⁸⁷
	Clamp at 40 mV						
	Ramp to -80 mV						

Table 1: Continued

Isoform	Type of protocol	Temperature (°C)	Ion used (K/Cs ⁺)	V _{1/2} (mV)	Slope	Current density in pA/pF	Cell type
Cardio-myocytes	-70 mV to 70 mV in 10 mV increments	37	Cs	/	/	-11.5 ± 1.8 at 50 mV	hESC-CMs ⁸⁸
	Tail at -80 mV						
	HP -40 mV	Physiological temperature	K	/	/	1.4 ± 0.2	iPSC-CMs ²⁴
	-50 to 30mV in 10 mV increments						
	Tail at -40 mV						
	HP -40 mV	Physiological temperature	K	/	/	0.7 ± 0.2	iPSC-CMs + shRNA
	-50 to 30 mV in 10 mV increments						against 1b) ²⁴
	Tail at -40 mV						iPSC-CMs ⁴¹
	HP -40 mV	36 ± 1	K	/	/	1.7	
	0 to 40 mV in 10 mV increments						
	Tail at -40 mV	37	K	/	/	0.75	iPSC-CMs ⁴⁹
	HP -50 mV						
	-40 to 40 mV in 10 mV increments						
	Tail at -50 mV						
	HP -80 mV	RT	Cs	-29.76 ± 3.31	/	6.6 ± 1.6 at -80 mV after depolarizing step to 20 mV	iPSC-CMs (this study)
	-70 to 70 mV in 10 mV increments						
	Tail at -80 mV						

Table 1: Continued

Isoform	Type of protocol	Temperature (°C)	Ion used (K/Cs)	$V_{1/2}$ (mV)	Slope	Current density in pA/pF	Cell type
	HP -80 mV	20-22	Cs	/	/	-16.5 ± 3.1	NRCMs ⁸⁰
	Depolarizing steps						
	Tail at -50 mV						
	HP -80 mV	RT	Cs	-38.9 ± 2.0	6.8 ± 0.3	12.5 ± 3.3 at -80 mV after depolarizing step to 20 mV	RVMs ⁷⁸
	-70 to 70 mV in 10 mV increments						
	Tail at -80 mV						
	HP -80 mV	23 ± 1	Cs	-41.7 ± 3.4	5.8 ± 0.3	17.9 ± 3.3 at -80 mV	NRCMs ⁸³
	-70 to 70 mV						
	Tail at -80 mV						
	HP -80 mV	23 ± 1	K	-6.2 ± 1.7	6.9 ± 1.5	3.9 ± 0.6	NRCMs ⁸³
	-70 to 70 mV						
	Tail at -80 mV						

NRCMs= neonatal rat ventricular myocytes; RVMs= rabbit ventricular myocytes

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Introduction

The pharmaceutical industry suffers from high attrition rates during clinical trials and after reaching the market, indicating a need for accurate predictive tools in early stages of drug development. However, current preclinical approaches involve simplified cellular assays or animal models that do not mimic human cardiac electrophysiology and are not accurate enough to detect possible drug-induced liabilities¹. More sophisticated preclinical tools have been proposed using human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) as an alternative to animal models. However, iPSC-CMs differ from human adult cardiomyocytes in structural and metabolic characteristics². Furthermore, iPSC-CMs have distinct electrophysiological features: a depolarized resting membrane potential (RMP) and uncontrolled automaticity, which can be explained due to the presence of the funny current, normally only present in nodal cells, and low levels of the inward rectifier potassium current I_{K1} ³. iPSC-CMs further lack T-tubules which compromises important protein-protein interactions for rapid and appropriately organized calcium handling⁴. I_{K1} is essential in this process, as I_{K1} finalizes repolarization, contributes to establishing a stable RMP and is important for other ion channel activity such as sodium and calcium³. Therefore we hypothesized in this thesis that *enhancing functional expression of I_{K1} in iPSC-CMs will generate a model that can be fully implemented in drug safety testing, and allows the replacement of currently used animal models with stem cell-derived models.*

State-of-the art molecular and computational techniques enabled us to modulate the functional expression of I_{K1} and improve the electrical properties of iPSC-CMs. We explain the current status of safety pharmacology and the potential for iPSC-CMs (Chapter 1), but also the drawbacks of the iPSC-CM electrophysiological phenotype (Chapter 2). *In silico* addition of I_{K1} highlights the importance of tailored models for automated patch clamp (Chapter 3) and single-cell patch clamp (Chapter 4). Overexpression of the I_{K1} channel protein (Chapter 5) highlights variability within iPSC-CM biology, which is further exemplified through the study of two patient-specific iPSC-CM lines with implications for precision medicine (Chapter 6). In addition, we focus on the unique features of the human Ether-à-go-go Related Gene (hERG) channel and the relevance for *in vitro* drug screening (Chapter 7).

Safety pharmacology

Cardiotoxicity screening is aimed at evaluating drug candidates for their potential proarrhythmic risks, which could lead to life-threatening conditions such as Torsade

de Pointes (TdP) arrhythmia. Blockage of the repolarizing rapid delayed rectifier potassium current I_{Kr} , conducted by the hERG channel, is considered as one of the main mechanisms driving drug-induced cardiotoxicity⁵. Therefore detecting I_{Kr} block has been at the foundation of safety pharmacology. The current guidelines on safety pharmacology include evaluation on a preclinical (S7B) and clinical (E14) level where the proarrhythmic risk is determined by the endpoints action potential prolongation and QT prolongation, respectively⁵. Implementation of these guidelines has increased the sensitivity (to accurately predict the proarrhythmic risk) of drug screening, since no torsadogenic drug has been withdrawn from the market after adoption of these guidelines⁶. However, the specificity (to accurately predict the negative proarrhythmic risk) of these guidelines is severely lacking, since potential new therapies may have been prevented from reaching the market⁷. The need to increase the specificity of safety pharmacology assays has a couple of years ago resulted in formation of consortia such as the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative that aim to refine preclinical drug screening. CiPA proposes a system in which: (1) drugs are tested on biological systems in which particular ion channels are overexpressed, (2) obtained data are confirmed by *in silico* simulation, (3) drugs are tested on iPSC-CMs and finally, (4) drugs are clinically evaluated⁵. The potential of the iPSC-CM as a proper preclinical model depends on the capability of the iPSC-CM to mimic the characteristics of the adult cardiomyocyte. However, the iPSC-CM electrophysiological phenotype is generally considered 'immature' as it resembles early embryonic rather than adult cardiomyocyte electrophysiology, which restricts their application in drug safety screening.

Is I_{K1} vital for iPSC-CMs?

In *Chapter 2* we put the immaturity of iPSC-CM electrophysiology into perspective and discuss its impact on drug safety screening. Recently, several other aspects of iPSC-CM electrophysiology have been addressed, such as the presence of acid-sensitive ion channels⁸ and non-cardiac channels as the big conductance calcium activated potassium current ($I_{BK,Ca}$) in iPSC-CMs⁹.

The concept of immaturity of iPSC-CMs and negating that immaturity has remained one of the trending topics within the field of iPSC-CMs. However, one of our findings was that studies into maturity often neglect I_{K1} electrophysiology, even though the lack of I_{K1} is specifically addressed through gene expression or protein quantification. Studies on maturity published after our findings often still do not address I_{K1} electrophysiology, but there are some exceptions. For instance, culturing iPSC-CMs

in multicellular preparations such as monolayers or 3D tissues has been suggested to increase I_{K1} density in iPSC-CMs^{10, 11}. In multicellular preparations the amount of cell-cell contacts increases, which in turn might promote the expression of ion channel proteins and potentiate the colocalization of ion channels¹⁰.

However, the lack of functional expression of I_{K1} in iPSC-CMs, and the subsequently anticipated influence on the RMP, has also been disputed and suggested to be the result of technical artefacts during patch clamp experiments¹¹. Indeed, recordings of resting membrane potential performed on small cells with high membrane resistances (e.g. iPSC-CMs) can underestimate the true RMP, which can be compensated for by using seal resistance compensation (Ahrens-Nicklas *et al.*¹² and *Chapter 3*). On the other hand, quantification of KCNJ2 mRNA consistently shows low expression levels in iPSC-CMs¹³. The role of I_{K1} in establishing the RMP was also challenged when specific block of I_{K1} by barium chloride ($BaCl_2$) prolonged action potentials but did not significantly alter RMP in healthy human ventricular cardiomyocytes¹⁴. This observation is in line with previous findings that outward potassium ion currents other than I_{K1} that play a role in the repolarization reserve (such as I_{Kr} and I_{Ks}) also contribute to the establishment of the RMP¹⁵.

Nevertheless, I demonstrate in this thesis that functional addition of I_{K1} does improve the electrophysiological characteristics of iPSC-CMs. The functional contribution of I_{K1} to iPSC-CM electrophysiology is signified by previous studies overexpressing I_{K1} which resulted in a higher upstroke velocity (due to increased sodium channel activation), more mature calcium handling¹⁶ and a better response to QT-prolonging drugs¹⁷.

Towards higher throughput for *in vitro* drug screening

Dynamic clamp (DC) is a technique in electrophysiology where a living cell is coupled to a mathematical model to inject virtual conductance in order to alter electrophysiological characteristics of e.g. cardiomyocytes¹⁸. Dynamic clamp thus allows precise control over the amount of current that is injected into the cell. Previously, *in silico* addition of I_{K1} has shown to hyperpolarize RMP, elicit human ventricular-like action potentials and decrease variability^{19, 20}. However, this process is manually executed and can only be applied to one cell at the time.

Manual patch clamp remains the gold standard for cellular electrophysiology, although it is laborious, requires specific technical skills and throughput is low. These issues might be circumvented by the use of automated patch clamp (APC) devices.

APC devices allow the measurement of multiple cells simultaneously, thereby decreasing the amount of manual labor, increasing the throughput and reducing variability²¹. Furthermore, automated patch clamp allows the screening of multiple compounds synchronously which is attractive for *in vitro* drug safety testing and thus the pharmaceutical industry. APC devices rely on chips with multiple small apertures to capture multiple cells within one experiment²². Cells are positioned on the aperture using suction, after which suction is also used to gain access to the interior of the cell.

In *Chapter 3* we describe a way to increase the throughput of single-cell electrophysiology experiments by combining APC with DC. Adding virtual I_{K1} resulted in a more hyperpolarized RMP, shorter action potentials, a restored plateau phase and improved drug response to calcium channel agonists and antagonists. Varying I_{K1} conductances can be used to influence action potential duration (APD) in iPSC-CMs which differed per cell, indicating the need for a tailored I_{K1} approach per cell. Since our study, several strides have been made to improve the recordings on APC devices. A general downside of APC in combination with iPSC-CMs is the relative lower quality of seals compared to manual patch clamp. The quality can be improved by using gentler reagents such as b-escin that do not rupture but perforate the cell membrane²³, changing the dissociation method of the iPSC-CMs²⁴ or incorporating a mechanism to compensate for the low seal resistance²⁵. Automated dynamic clamp has recently been extended to 8-channels, further increasing the throughput²⁶.

APC devices have been on the market for more than 15 years and are now routinely being applied by academia and industry, while development also takes place to increase the throughput of these platforms²¹. APC devices can be used for various drug discovery and screening purposes such as ligand discovery, mechanism of action and structure-function studies²⁷. This has been particularly useful in drug discovery for pain-related ion channels, where APC devices have aided in developing new drugs that ultimately entered clinical trials²⁸. APC devices are envisioned to be used in initiatives such as CiPA in the future for high-throughput drug screening²⁹.

The critical amount of I_{K1} (*in silico*)

The effect of I_{K1} addition through dynamic clamp relies heavily on the I_{K1} formulation that is being used. In *Chapter 4* we describe the influence of different *in silico* I_{K1} formulations on simulated APs in iPSC-CM models. We analyzed six I_{K1} formulations for their current-voltage characteristics and AP waveform properties at varying conductances. There are large differences in the way the available models describe the behavior of I_{K1} channels, especially at potentials depolarized from the RMP such

as during the plateau phase of the AP. We show that depolarizing and repolarizing prepulses before the pacing stimulus only lead to AP prolongation or shortening respectively in the iPSC-CM model with one (Ishihara) of the six formulations. The Ishihara formulation is the only formulation to incorporate the time-dependent properties of the I_{K1} channel, which are important to consider in drug screening purposes. Therefore we conclude that the Ishihara formulation might work best in DC experiments. We also introduced the concept of a cell-specific critical value of I_{K1} conductance ($G_{K1, \text{critical}}$), which is the value of I_{K1} that is required to stop automaticity in iPSC-CMs. With including the $G_{K1, \text{critical}}$ in higher throughput (APC) systems we will be able to optimize the electrical characteristics per cell, which will ultimately decrease variability between iPSC-CMs and improve the predictive quality of drug safety assays.

The choice for a particular model or formulation is not only of importance in dynamic- and automated dynamic clamp experiments. In CiPA, one of the pillars consists of confirming drug effects *in silico* (phase 2) in a human ventricular cardiomyocyte model to aid in proarrhythmic risk prediction. Two important aspects are currently hampering: a clear strategy on which proarrhythmic endpoints and predictive criteria need to be included, and which drugs will be used to train and validate the model to standardize *in silico* measurements for all users. The second aspect is how to capture the heterogeneity of the human population and how to apply proper statistical methods^{30, 31}. For cardiotoxicity screening in the context of cardiac disease, the impact of disease state on the model is an important aspect to consider as well. Cardiac diseases influence ion channel expression, which in turn can affect action potential duration and change drug response³². For the detection of the proarrhythmic risk of drugs, disease state might therefore prove vital for *in silico* predictions.

The critical amount of I_{K1} (*in vitro*)

As we have demonstrated, the application of dynamic clamp influences iPSC-CM electrophysiology. It is not clear to which extent *in silico* current might change ion channel trafficking or signaling processes within the cell. The biological consequences will likely be negligible during an experiment, since dynamic clamp experiments are relatively short-lived. In order to study the effect of varying I_{K1} conductances *in vitro*, we applied a biological approach to add I_{K1} to iPSC-CMs. Therefore we increased I_{K1} current density by overexpressing the KCNJ2 gene, coding for the Kir2.1 ion channel protein, in iPSC-CMs in *Chapter 5*. In our proof-of-concept we observed very negative RMPs, approaching the potassium equilibrium potential E_K , and leading to absolute

arrest of spontaneous activity after KCNJ2 transfection. This quiescence could be mitigated by activating the optogenetic sensor ChR2 to trigger action potentials by light. ChR2 generated AP-like signals and allowed pacing by light. The critical amount of I_{K1} necessary to hyperpolarize the cell differed per cell which illustrated, in conjunction with Chapters 3 and 4, the biological variability within iPSC-CMs and the need for a tailored cell-specific approach.

Next to overexpression of the KCNJ2 gene we evaluated other measures to increase I_{K1} current density biologically. Inhibition of microRNA-1 (miR-1) has been postulated to increase KCNJ2 expression and, consequently, I_{K1} current density in ventricular cardiomyocytes^{33, 34}. We hypothesized that knock-down of miR-1 could increase KCNJ2 and thus I_{K1} current density in iPSC-CMs. Therefore we transfected iPSC-CMs with a miR-1 antagonist, antagomiR-1, using a lipid/polymer formulation-based reagent suited for small RNAs and subsequently analyzed RNA expression with PCR. We were successful in decreasing miR-1 expression in iPSC-CMs, though that did not result in higher KCNJ2 expression in our iPSC-CMs (Figure 1). miR-1 might have a dual role, as miR-1 transduction has also been associated with shorter APD and hyperpolarized RMP due to upregulation of other potassium currents (I_{to} , I_{Ks} and I_{Kr})³⁵.

In another pilot study we cocultured iPSC-CMs with I_{K1} -producing Human Embryonic Kidney (HEK) cells, which was hypothesized to increase I_{K1} current density in iPSC-CMs through electrical coupling mediated via gap junctions. We cultured iPSC-CMs with Ex293 cells, which are HEK cells stably expressing sodium channel protein $Na_v1.5$, Kir2.1 and gap junction protein connexin43. We seeded cells in different ratios (1:1, 1:3 and 1:10) in polydimethylsiloxane (PDMS) molds coated with extracellular matrix-like proteins. After several days in culture we analyzed the action potential characteristics with voltage-sensitive dyes. Cocultures of iPSC-CMs with Ex293 cells displayed quiescence, lower beats per minute (BPM) and prolonged APDs. The beating rate decreased from 51 ± 5 BPM in monocultures of iPSC-CMs to 44 ± 8 BPM in cocultures, while the APD₉₀, the action potential duration at 90% of repolarization, increased from 108 ± 18 ms in monocultures of iPSC-CMs to 449 ± 26 ms in cocultures of iPSC-CMs. These findings are in line with previous studies, where cocultures reduced automaticity³⁶ and prolonged action potential duration³⁷. However, coculturing iPSC-CMs with other cell types introduced several practical limitations. The main issue was controlling the ratio of iPSC-CMs to HEK cells and influencing the type of cell-cell connections. Since HEK cells divide, in contrast to iPSC-CMs, we needed to slow HEK cell growth in order to create the desired ratios of iPSC-CMs to HEK cells.

Figure 1: Expression of miR-1 and KCNJ2 in transfected iPSC-CMs.
The RNA expression of miR-1 and KCNJ2 was measured in three different lines of iPSC-CMs (A-C), which were either untransfected (wt), or transfected with different concentrations of transfection reagent and DNA or scramble (1-11 with and without s). Amount of DNA to transfection reagent in ml: 1:4:1.66 (1), 2:1.66 (2), 1:4:1.66 (scramble) (3(s)), 1:2:5 (4), 1:4:2:5 (5), 2:2:5 (6), 1:4:2:5 (scramble) (7(s)), 1:5 (8), 1:4:5 (9), 2:5 (10), 1:4:5 (scramble) (11(s)). Results were normalized against housekeeping gene TBP. Values at each concentration represent the average of a technical replicate $\pm$ SEM.

However, we did not find the right balance between cell death and growth arrest in HEK cells, thus complicating tightly regulated cocultures. Furthermore, in the cocultures that were not overgrown by HEK cells, iPSC-CMs preferred to connect to other iPSC-CMs instead of HEK cells. For future studies we propose to employ sophisticated techniques such as microfluidics to control the patterning of certain geometries and the amount of cells³⁸.

Both *in silico* and *in vitro* approaches enabled hyperpolarization of iPSC-CMs and showed the relevance of I_{K1} balance which contributed to electrical maturation of iPSC-CMs. Whether full maturation will be achieved using these 'cosmetic' approaches post-differentiation, remains to be determined since the process of maturation *in vivo* itself is not yet completely clear. There is plenty of information on the embryonic development of the heart as well as the early postnatal heart, but for further knowledge we mostly build on animal models³⁹. In order to nudge iPSC-CMs towards mature cardiomyocytes, we need to study maturation ante- and postnatally *in vivo* to truly capture the signaling and stimulation cues that are necessary to promote maturity in culture^{40, 41}. Currently, culturing iPSC-CMs in engineered heart tissues (EHTs) seems to yield the most 'mature' cardiomyocytes, as EHTs in the most optimal way allow to mimic the cell-cell connections and environment in 3D, allowing electrical and mechanical loading of the embedded cardiomyocytes³⁹.

Disease modeling revisited

iPSC-CMs may not only be suitable for *in vitro* drug screening, but may also become a platform for patient-specific disease modeling. It is envisioned that disease mechanisms can be studied, as well as screening for patient-specific drugs, also called precision medicine, enabling the right medication for each patient. In *Chapter 6* we screened two iPSC-CM lines derived from patients suffering from arrhythmogenic cardiomyopathy, for experimental compounds and described the electrophysiological read-out. In both lines we showed that the iPSC-CM lines partially but not fully recapitulate the electrophysiological phenotype as anticipated, but drug response was not as compared to other iPSC-CM lines or animal models. This aligns with previous findings, where the disconnect between data derived from iPSC-CMs and the clinical phenotype is attributed to the well-known immaturity and heterogeneity of iPSC-CMs⁴². This variability and heterogeneity in iPSC-CMs can be caused through natural genetic and epigenetic variation, the somatic cell source, pluripotency protocols, differentiation protocols, the efficiency of differentiation, and culture or maturation approaches⁴³. How to decrease the variability depends on

the context of use and the desired endpoint. For disease modeling, the main goal is to distinguish healthy from diseased without natural biological variation biasing interpretation. Recent studies indicate that the amount of individuals⁴⁴ and the choice for proper controls⁴⁵ improve the power of the disease under study. Indeed, we also found the importance of using genetically matched controls for elucidating the mechanism of disease *in vitro*. We also demonstrated the power of including a CRISPR-induced 'reverted' iPSC-CM phenotype in disease modeling, which serves as an additional control.

Caution concerning hERG in CiPA

In safety pharmacology, block of the I_{Kr} (hERG) channel is of interest because of the role of I_{Kr} in action potential duration and the high propensity of many drugs towards block of the channels conducting I_{Kr} . In *Chapter 7* we discuss the different isoforms of hERG in cardiomyocytes which influence electrophysiology and drug response and show alternative methods to isolate I_{Kr} in patch clamp experiments.

hERG block is often associated with torsadogenic endpoints, although hERG block might not be the best predictor for proarrhythmia^{46, 47}. There are several other predictors such as triangulation⁷ and beat-to-beat variability⁴⁸ which might associate well with a proarrhythmia score as well. Currently, there are no clear endpoints defined for *in vitro* drug screening initiatives such as CiPA. Endpoints vary between repolarization-associated predictors⁴⁹, TdP incidence and conduction slowing⁵⁰ and contractile parameters^{2, 51}. The lack of consistency in endpoint definition contributes to the large variability in CiPA studies using iPSC-CMs. Furthermore, these endpoints are mainly acute which suits well in drug screening studies that aim at detecting the short-term effects of drugs. However, when screening for chronic effects, long-term changes such as ion channel trafficking or contractile remodeling⁵² should be considered as well.

Conclusion

In this thesis we show that adding I_{K1} to iPSC-CMs increases their electrical resemblance to adult CMs in terms of hyperpolarized properties, though we do not consider I_{K1} addition to be the holy grail for iPSC-CM maturity. Tweaking only one characteristic of iPSC-CMs will ameliorate that characteristic but not all, and a holistic approach might be needed to generate a true adult electrical phenotype. Additionally, there is a gap in knowledge on multiple aspects of cardiac maturation

in vivo, which hampers the current approaches to realize a fully mature iPSC-CM model *in vitro*.

Whether iPSC-CMs will be able to replace animals in preclinical drug screens or whether it will serve as an additional preclinical tool before proceeding to animal studies, depends on the context in which iPSC-CMs will be used and which flaws we are willing to accept. The current field of iPSC-CMs raises more questions than it answers, and needs a clear consensus on endpoints regarding maturity and proarrhythmia. We have made tremendous strides in the field of stem cells and technology, but we may just need to pace ourselves in order to move forward.

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Nederlandse samenvatting

Introductie

Het ontwikkelen van nieuwe medicijnen kost veel tijd en geld. Daarnaast worden weinig medicijnen goedgekeurd omdat ze ongunstige bijwerkingen veroorzaken, of worden ze om die reden teruggetrokken als ze eenmaal op de markt zijn. 45% van de teruggetrokken medicijnen veroorzaakt de zeldzame en mogelijk dodelijke hartritmestoornis Torsade de Pointes (TdP), waardoor het nu verplicht is geworden om medicijnen in ontwikkeling te testen op bijwerkingen op het hart. Bij voorkeur gebeurt dit op menselijk hartweefsel, maar dit is lastig te verkrijgen en moeilijk in kweek te houden. Er wordt dan ook veel gebruik gemaakt van dierlijk materiaal bij de ontwikkeling van nieuwe medicijnen, ook al kunnen dieren anders op medicijnen reageren dan mensen. Het testen op dierlijk materiaal kan daarom leiden tot verkeerde voorspellingen over de veiligheid van nieuwe medicijnen.

Het ontwikkelen van proefdiervrije (*in vitro*) methoden, die meer op mensen gericht zijn, heeft internationaal veel aanzien gekregen en is vastgelegd in het Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiatief. Dit wereldwijde consortium is gebaseerd op het testen van medicijnen met behulp van humane stamcel-afgeleide hartspiercellen (induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs)). iPSC-CMs zijn somatische cellen, bijvoorbeeld uit de huid of urine, die zijn gereprogrammeerd tot stamcellen en gediifferentieerd tot hartspiercellen. Op deze manier is het in theorie mogelijk om van ieder individu een oneindige hoeveelheid hartspiercellen te maken, wat de ontwikkeling van proefdiervrije methoden kan bevorderen.

Het is daarvoor belangrijk dat iPSC-CMs op volwassen hartspiercellen lijken. Normaliter trekt de volwassen hartspiercel samen als gevolg van de in- en uitstroom van ionen zoals natrium, calcium en kalium. Door een positief verschil in voltage, of potentiaal, wordt de hartspiercel geactiveerd (depolarisatie). Als gevolg openen natriumkanalen voor een inwaartse natriumstroom. Natriumkanalen inactiveren en een kaliumkanaal opent kortstondig voor een kleine uitwaartse kaliumstroom. Vervolgens openen calciumkanalen voor een inwaartse calciumstroom, die op hun beurt voor nog meer calciumafgifte vanuit de cel zorgen. De cel kan nu samentrekken. Vervolgens openen kaliumkanalen voor een uitwaartse kaliumstroom om activatie te beëindigen (hyperpolarisatie). De hartspiercel komt hiermee in een ruststand op een negatief voltage, de zogenaamde rustmembraanpotentiaal (RMP).

iPSC-CMs bevatten de meeste ionstromen, maar bevatten ook een activerende stroom die normaal alleen voorkomt in de cellen van de sinusknoop. Daarnaast werkt de calciumhuishouding niet optimaal en ontbreekt het aan de inwaarts rectificerende kaliumstroom (I_{K1}). Door deze verschillen in elektrische eigenschappen is het RMP van iPSC-CMs positiever, of lichtelijk gedepolariseerd, en zijn de cellen spontaan actief. Ze lijken hierdoor meer op embryonale hartspiercellen dan op volwassen hartspiercellen en worden daardoor vaak 'foetaal' of 'immatuur' genoemd. Door de verschillen in elektrische eigenschappen reageren iPSC-CMs anders op medicijnen dan volwassen hartspiercellen. Ze zijn daardoor op dit moment niet geschikt voor het gebruik als proefdiervrije methode om de bijwerkingen van medicijnen te testen.

I_{K1} is binnen de hartspiercel verantwoordelijk voor het behouden van een negatief RMP. Als we I_{K1} toevoegen, zal het RMP negatiever worden, en we verwachten dat dit voldoende kan zijn om de spontane activiteit te onderdrukken en voor stabielere elektrische eigenschappen te zorgen. In dit proefschrift introduceren we I_{K1} in iPSC-CMs via verschillende manieren met als doel een beter model te maken voor het proefdiervrij testen van (nieuwe) medicijnen op bijwerkingen op het hart.

Dit proefschrift

In **hoofdstuk 1** leggen we de activatie van het hart uit van hartspiercel tot orgaan, alsmede het proces van medicijnontwikkeling en de verschillen tussen mens en dier.

Hoofdstuk 2 bevat een literatuurstudie waarin we beschrijven hoe belangrijk I_{K1} is voor het functioneren van de hartspiercel en dus ook voor de iPSC-CM. I_{K1} beïnvloedt namelijk ook de werking van andere eiwitten en ionstromen en is daarmee bepalend voor de activiteit van de hartspiercel. De invloed van I_{K1} op de natriumstroom (I_{Na}) is verder uitgewerkt in het **addendum van hoofdstuk 2**. Verder geven we een overzicht van studies die het gebrek aan I_{K1} in iPSC-CMs aanpakken en het effect van die aanpak op de elektrische eigenschappen van iPSC-CMs.

De elektrische activiteit van één hartspiercel kan nauwkeurig gemeten worden door middel van patch clamp; een techniek die veel expertise en tijd vereist en daardoor niet geschikt is voor medicijnonderzoek op grote schaal. Inmiddels zijn er ook apparaten die automatisch patchen door middel van robotica, en meerdere cellen tegelijk kunnen meten. In **hoofdstuk 3** combineren we automatische patch clamp met dynamic clamp, waarbij een hartspiercel aan een I_{K1} computermodel wordt gekoppeld om virtueel I_{K1} te injecteren. We zijn erin geslaagd om deze twee technieken met elkaar te combineren en zo meerdere iPSC-CMs tegelijk te meten. Door te variëren in de hoeveelheid I_{K1} kanalen (conductantie, G_{K1}) ontstaan

er verschillen in actiepotentiaalvorm en -duur. Het toevoegen van I_{K1} in iPSC-CMs zorgt voor een verbetering van natriumkanaalfunctie (snellere upstroke velocity) en kortere actiepotentialen. Meer I_{K1} zorgt daarnaast voor een hogere gevoeligheid voor het effect van calcium (ant)agonisten, wat aantoont wat voor toegevoegde waarde I_{K1} heeft voor de iPSC-CM als model om medicijnen op te testen.

In **hoofdstuk 4** bouwen we verder op dynamic clamp. Er zijn namelijk verschillende computermodellen beschikbaar om I_{K1} te injecteren in dynamic clamp experimenten, maar die variëren in hoeverre ze de werking van het daadwerkelijke ionkanaal kunnen nabootsen. In dit hoofdstuk vergelijken we vijf I_{K1} computermodellen en een iPSC-CM computermodel met als doel te bepalen welke het beste gebruikt kan worden voor dynamic clamp experimenten. Hiervoor hebben we de $G_{K1\text{critical}}$ geïdentificeerd: de minimale I_{K1} conductantie waarbij een iPSC-CM een stabiel en voldoende negatief (gehyperpolariseerd) RMP heeft en spontane activiteit onderdrukt wordt. De $G_{K1\text{critical}}$ kan vervolgens gebruikt worden als referentiewaarde tijdens dynamic clamp experimenten. We concluderen dat het Ishihara I_{K1} model het meest geschikt is, omdat deze het beste de tijdsafhankelijkheid van het ionkanaal nabootst. *In vitro* experimenten met iPSC-CMs laten zien dat er veel variabiliteit is binnen één cellijn en dat elke cel een andere hoeveelheid I_{K1} nodig heeft.

Een balans in de hoeveelheid I_{K1} is zeer belangrijk; te weinig I_{K1} is onvoldoende om de spontane activiteit te onderdrukken, te veel I_{K1} zorgt ervoor dat de hartspiercel niet meer geactiveerd wordt. In **hoofdstuk 5** transfecteren we het gen coderend voor het I_{K1} kanaaleiwit in iPSC-CMs om ze te hyperpolariseren, alsmede het gen voor een ionkanaal dat activeert na stimulatie met licht om de iPSC-CMs te kunnen depolariseren. We hebben verschillende verhoudingen van de ionkanalen getransfecteerd om het effect op actiepotentiaalduur en -vorm te bepalen. Hoe meer I_{K1} er toegevoegd werd, hoe negatiever het RMP werd en hoe korter de actiepotentialen werden. Net als in **hoofdstuk 4** observeren we verschillen in hoe veel I_{K1} nodig is; sommige iPSC-CMs hadden weinig I_{K1} nodig om stabiel te worden, terwijl sommigen na heel veel I_{K1} nog steeds spontaan actief waren. Het geeft aan dat elke iPSC-CM een I_{K1} aanpak op maat nodig heeft.

iPSC-CMs kunnen, naast het testen van medicijnen, potentieel ook dienen als model om *in vitro* hartritmestoornissen na te bootsen. In **hoofdstuk 6** meten we de elektrische activiteit van iPSC-CMs van twee patiënten met de erfelijke hartritmestoornis Aritmogene Cardiomyopathie (ACM). In **deel A** bestuderen we iPSC-CMs van een ACM-patiënt met een nieuwe mutatie in desmoplakine, een eiwit dat

belangrijk is voor de connecties tussen individuele hartspiercellen. We observeren afwijkingen in actiepotentiaalduur (langer) en calciumsignalen (kleiner) vergeleken met controle iPSC-CMs, wat in lijn ligt met het ziektebeeld van ACM. In **deel B** bestuderen we iPSC-CMs van een ACM-patiënt met een mutatie in phospholamban (PLN), een eiwit dat belangrijk is voor het vrijkomen en de heropname van calcium in de hartspiercel. We observeren afwijkingen in de calciumsignalen (kleiner en trager), vergelijkbaar met eerdere studies wat indicatief is voor het ziektebeeld. Toevoeging van het experimentele middel istaroxime, bedoeld om de abnormale calciumhuishouding te verbeteren, had tegenstrijdige resultaten ten opzichte van eerdere studies. Daarnaast observeren we in zowel deel A als B, vergelijkbaar met **hoofdstuk 4 en 5**, grote variabiliteit tussen de iPSC-CMs van dezelfde patiënt. Dit wordt grotendeels veroorzaakt door natuurlijke biologische verschillen, maar ook door de manier waarop de iPSC-CMs worden gereprogrammeerd tot stamcellen en gedifferentieerd tot hartspiercellen. Het is hierdoor lastig om conclusies te trekken over het specifieke mechanisme van een bepaald ziektebeeld.

In **hoofdstuk 7** concentreren we ons op een andere kaliumstroom: I_{Kr} (hERG), wat een belangrijk target is in medicijnonderzoek. Veel medicijnen grijpen namelijk aan op I_{Kr} , en blokkade van deze stroom kan bijdragen aan het ontstaan van hartritmestoornissen zoals TdP. Om het effect van medicijnen op deze stroom te testen, kan hERG tot expressie gebracht worden in een cellijn. Het hERG-kanaal bestaat in hartspiercellen uit de isovormen 1a en 1b, maar voor het testen van medicijnen in cellijnen wordt meestal alleen hERG1a tot expressie gebracht. In dit hoofdstuk meten we I_{Kr} kinetiek in iPSC-CMs met patch clamp om te kijken welke isovorm domineert in deze cellen. We observeren dat I_{Kr} kinetiek in iPSC-CMs meer lijkt op dat van hERG1b, wat gevolgen kan hebben voor de respons op medicijnen. Het niet includeren van hERG1b in cellijnen voor medicijnonderzoek zorgt voor een incompleet beeld van de I_{Kr} kinetiek, en kan leiden tot verkeerde voorspellingen over de veiligheid van medicamenten.

In **hoofdstuk 8** verbinden we alle punten binnen de hoofdstukken in een algemene discussie en bespreken we pilotexperimenten die niet zijn uitgemond in een volledig project.

Conclusie

In dit proefschrift zijn we erin geslaagd om met verschillende technieken I_{K1} toe te voegen aan iPSC-CMs. Het zorgt voor stabielere elektrische eigenschappen, zoals een gehyperpolariseerd rustmembraanpotentiaal en minder spontane activiteit. Maar, elke hartspiercel heeft een andere hoeveelheid I_{K1} nodig en het toevoegen

van I_{K1} verbetert niet alle elektrische eigenschappen van iPSC-CMs. Het gebrek aan standaardisatie van differentiatie naar iPSC-CMs en het gebrek aan richtlijnen omtrent het gebruik van iPSC-CMs vormen aanvullende obstakels voor de overgang naar proefdiervrij medicijnonderzoek. Er zijn veel stappen gezet op weg naar het proefdiervrij testen van medicijnen wat betreft stamcellen en technologie, maar er liggen nog veel vragen die beantwoord moeten worden. Het veld ontwikkelt zich in een rap tempo, maar misschien moeten we wel een pas op de plaats maken in plaats van vooruit te blijven hollen.

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Woonzorgcentrum De Drie Ringen heeft een speciale plek in mijn leven. **Sandra**, dankjewel voor het zoeken naar een geschikte vrijwilligersfunctie voor mij, je reguliere contact over hoe het gaat en voor je interesse in mijn promotietraject. **Daya**, samen met jou yoga geven is altijd hilarisch, dank voor je continue vrolijkheid! Ik

heb veel respect voor hoe jij het welzijnsprogramma inricht en ik zie hoe dat (en jij) gewaardeerd wordt door de bewoners.

De afgelopen vier jaar heb ik met heel veel plezier gesport bij The Bootcamp Club, wat voor mij een bron was voor ontlading en ontspanning. Ik werd continu gemotiveerd om sterker en fitter te worden door de kundige trainers, met in het bijzonder **Jasper, Marcel, Koos, Martijn en Ali. Daan**, dank voor het gepersonaliseerde sportschema, je interesse in mijn promotietraject en je humor tijdens de training. Daarnaast heb ik ook een mooie groep overgehouden aan het boksen. **Boksende borrelaars**, zo heten we inmiddels toch? Ik hoop dat we snel weer een zelfgemaakt zesgangendiner met bijpassend wijnarrangement kunnen organiseren. **Yldou**, dank voor alle gezelligheid tijdens en buiten het sporten! **Marije**, fijn dat we als duo crossfit hebben geprobeerd, die box was samen toch minder eng. **Saskia**, je bent een ontzettend motiverend sportmaatje, dank daarvoor! Weliswaar als het niet regent en je kleding niet in de was zit. **Susanna** je energie en lach zijn aanstekelijk, en zijn altijd een bron voor gezelligheid. Dank voor de ritjes naar het boksen, het samen sporten, en de wandelingen waar we alles konden bespreken. **Hester**, er zijn tijden geweest dat onze weekindeling om het boksen heen werd gepland, met als ultiem hoogtepunt een zelf-georganiseerde bokstraining mét cooling down op Mr Bombastic. Wat hebben we fijn gepartnerd tijdens het boksen, en ik vind het prettig dat we nu nog regelmatig zo veel delen! Van wandelingen (met of zonder Milo) tot ons bescheuren bij Showponies ('korfgenoten...'). Dankjewel voor alles.

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zou bespelen, welke zou dat zijn?' niet vreemd zijn in onze gesprekken. Dankjewel voor alles! **Elise** (Hovingh), mijn hersengenoot! Soms is het eng hoe vaak we op één lijn zitten. Van rare Nederlandse gebruiken, tot communicatie (op de werkvloer) en duurzamer leven en nog veel meer. Ik houd van je rationele houding en je vrolijkheid en dat we alles kunnen delen. Ik vind je zo'n ontzettend sterke vrouw (en leider in wording) en ik heb veel bewondering voor je! **Nathalie**, onze vriendschap is altijd stabiel en dat is fijn. Je houdt van aanpakken, zegt duidelijk waar het op staat – zeker als het onrecht betreft – en dat vind ik een van je beste kwaliteiten, alsmede een onuitputtelijke bron voor goede verhalen. We kunnen een boek schrijven over al onze uitspraken en bijnamen die we ooit bedacht hebben, maar ook over de avonturen op vakantie. Ik noem een achtervolging in de Parijse metro, een mountainbiketocht in Noorwegen die we voornamelijk gelopen hebben, of hoe we de berg over "gerausd" zijn in Oostenrijk. Met jou is het nooit saai en ik kijk uit naar onze volgende reis samen.

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List of publications

Publications in this thesis:

Fabbri, A., **Goversen, B.**, Vos, M.A., van Veen, T.A.B., de Boer, T.P. Required G_{K1} to suppress automaticity in iPSC-CMs depends strongly on I_{K1} model structure. *Biophysical Journal* **117**, 1–13 (2019).

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Goversen, B.*, Becker, N.*, Stoelzle-Feix, S., Obergrussberger, A., van Veen, T.A.B., Fertig, N., de Boer, T.P. Hybrid models for safety pharmacology on an automated patch clamp platform: joining iPSC-derived cardiomyocytes and simulations of I_{K1} ion channels in real-time. *Frontiers in Physiology* **8(1094)**, 1–10 (2018).

Goversen, B., van der Heyden, M.A.G., van Veen, T.A.B., de Boer, T.P. The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on I_{K1} . *Pharmacology & Therapeutics* **183**, 127–136 (2018).

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* These authors contributed equally

Other publications:

Poldervaart, M.T., **Goversen, B.**, de Ruijter, M., Abbadessa, A., Melchels, F.P.W., Öner, F.C., Dhert, W.J.A., Vermonden, T., Alblas, J. 3D bioprinting of methacrylated hyaluronic acid (MeHA) hydrogel with intrinsic osteogenicity. *PLoS One* **12(6)**, 1–15 (2017)

Curriculum Vitae

Birgit was born on the 9th of July 1990 in Kaatsheuvel, and grew up in Tilburg. She followed her pre-university education at Mill Hill College in Goirle and knew from an early age she wanted to study Biomedical Sciences at Utrecht University, which she started in 2008. After her bachelor studies, Birgit became a board member of the study association Medisch Biologen Vereniging Mebiose where she coordinated student participation for both the bachelor and master Biomedical Sciences. She continued her studies with the master programme Regenerative Medicine and Technology at Utrecht University in 2012, which ignited her interest for developing *in vitro* alternatives to animal testing. This is reflected by internships on bone tissue engineering (Department of Orthopedics, UMC Utrecht) and on cartilage tissue engineering (Department of Biomedical Engineering, Eindhoven University of Technology). She also developed workshops for teachers on how to implement more *in vitro* research into their education (Institute for Life Sciences & Chemistry, University of Applied Sciences Utrecht). After her studies, Birgit applied for a PhD position and found a match with the Department of Medical Physiology at the UMC Utrecht. In her PhD, she employed various techniques to improve the electrical characteristics of induced pluripotent stem cell-derived cardiomyocytes for *in vitro* drug screening. The results are presented in this thesis. During her PhD, Birgit also fostered her interest for teaching by obtaining her Basiskwalificatie Onderwijs (BKO).

amplifier

level 1

level 2

level 3

filter

high-pass

low-pass

comb

notch

band-pass

miscellaneous

I_{NCX}

$I_{to,s}$

$I_{Ca,T}$

I_{KATP}

calcium

RyR

PLN

IP_3R

PKA

PKC

CaM

CaMKII

PP1