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The immature electrophysiological phenotype of iPSC-CMs still hampers *in vitro* drug screening: Special focus on I_{K1}

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ABSTRACT

Preclinical drug screens are not based on human physiology, possibly complicating predictions on cardiotoxicity. Drug screening can be humanised 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 hyperpolarising current I_{K1}. In adult cardiomyocytes, I_{K1} finalises repolarisation by stabilising 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 responses. 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.

1. 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 overexpression cell lines, isolated cardiomyocytes, isolated animal hearts and *in vivo* animal testing (Jonsson et al., 2009). 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 death (Ferri, 2013). 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 repolarisation in cardiomyocytes. This has led to release of the International Conference on Harmonisation 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 incidence (Bossu, van der Heyden, de Boer, & Vos, 2016). 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 arrhythmias (Bossu et al., 2016).

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 composition (O'Hara, Virág, Varró, & Rudy, 2011). 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 assay (see Gintant, 2011), although performance can be improved by implementation of drug effects on multiple ion channels in computer simulations of cardiac action potentials (Mirams et al., 2014). 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) initiative (Gintant, Sager, & Stockbridge, 2016). 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

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good resemblance between iPSC-CM electrophysiology and adult cardiomyocyte electrophysiology.

1.1. 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 depolarising stimulus to fire an action potential, as adult cardiomyocytes of the working myocardium do. The automaticity is the result of an interplay between depolarising and repolarising ion currents. iPSC-CMs are characterised by presence of the funny current (I_f), which is only significantly present in cells in the sinoatrial node of the adult heart (Verkerk et al., 2007) to drive pacemaking. iPSC-CMs also display very low levels of the inward rectifier potassium current (I_{K1}), which stabilises the resting membrane potential (RMP) in adult cardiomyocytes and is responsible for the final phase of repolarisation (Dhamoon & Jalife, 2005). 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 mV (Hoekstra, Mummery, Wilde, Bezzina, & Verkerk, 2012)), allowing the depolarising 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 pacemaking (Sun et al., 2017). 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 safety (Bossu et al., 2016). Therefore, the presence of I_f and absence of I_{K1} cause proarrhythmic traits in iPSC-CMs that have to be considered carefully (Jonsson et al., 2012). 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.

2. 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 depolarising stimulus arrives, voltage-gated sodium channels open to allow influx of sodium (phase 0). After a small repolarisation, voltage-gated calcium channels open to allow influx of calcium *via* L-type calcium channels, and evoke calcium-induced calcium release from the sarcoplasmatic reticulum (SR) giving rise to the plateau phase (phase 2). Then the I_{Kr} channels open to facilitate repolarisation (phase 3), after which I_{K1} stabilises 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 depolarised Fig. 1A. A depolarised resting membrane potential causes sodium channels, which depend on voltage, to remain inactive (Rook et al., 1999). 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 hyperpolarised 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.

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 mV (Dhamoon & Jalife, 2005). 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 depolarisation. This feature, referred to as rectification, is caused by blocking of the channel's inner pore by magnesium, spermine or spermidine at depolarising membrane potentials (Huang & Kuo, 2016). 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} (De Boer, Houtman, Compier, & Van Der Heyden, 2010). The consequences have been also demonstrated in transgenic mice harbouring 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 hearts (McLerie & Lopatin, 2003). A complete knockout of KCNJ2 in mice led to mortality between 8 and 12 h after birth due to a cleft palate (Zaritsky, Redell, Tempel, & Schwarz, 2001). 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 dysmorphology (Plaster et al., 2001).

A gain-of-function in I_{K1} has been associated with Short QT Syndrome and familiar 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 arrhythmia (Priori et al., 2005). 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 (Li, Mclerie, Lopatin, Mclerie, & Lopatin, 2004, Qi et al., 2015). The examples in human disease underline the importance of I_{K1} current density to maintain normal cardiac excitability and rhythm.

2.1. 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 Kir2.2 (Schram, Pourrier, Wang, White, & Nattel, 2003). Hetero-tetramers have altered gating and conductance (Panama, Mclerie, & Lopatin, 2010). Kir2.1 is mainly localised in the ventricle and is less abundant in the atria, leading to a reduced I_{K1} current density in the atria (Melnyk, Zhang, Shrier, & Nattel, 2002). The expression differences between tissues are mainly controlled by transcriptional regulation of the aforementioned KCNJ2 gene (Redell & Tempel, 1998). 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 transcription (Redell & Tempel, 1998). 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).

2.2. 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 Kir2.1 (Houtman et al., 2014). However, the

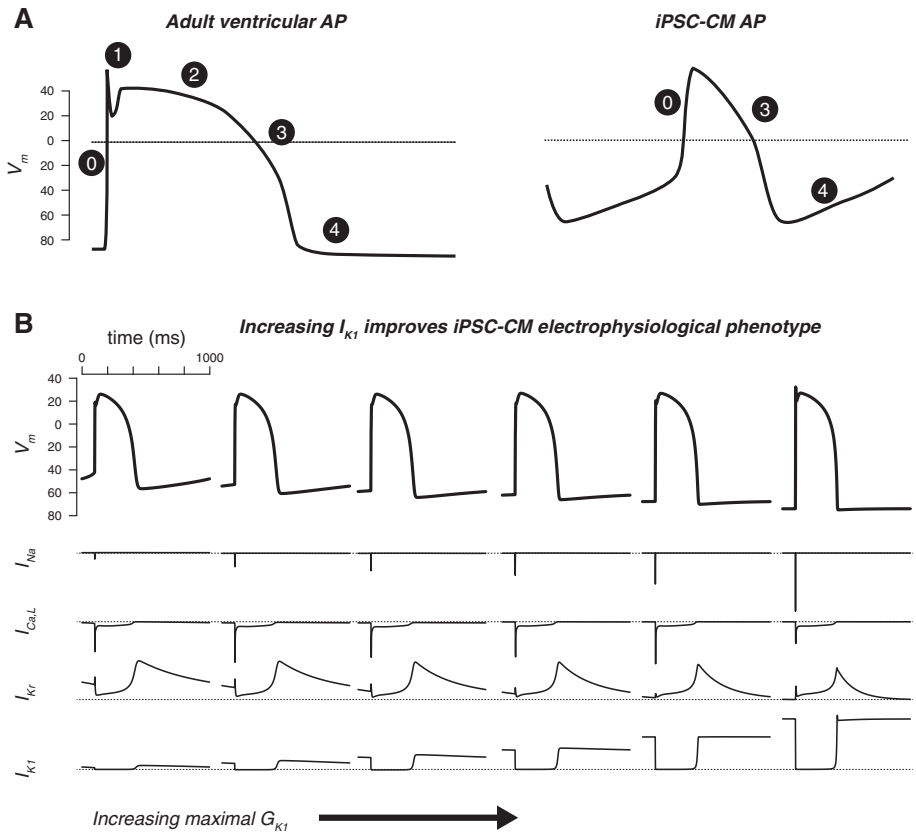

Fig. 1. A. Schematic depicting the differences between the action potential waveform of human adult ventricular cardiomyocytes and that of human iPSC-CMs. Numbers indicate the various phases of the action potential. Note the spontaneous depolarisation during phase 4 and the slow upstroke velocity of phase 0 in 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 the repolarisation (phase 3) of the action potential.

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 Kir2.1 (De Boer et al., 2010). Moreover, in skeletal myoblasts, hyperpolarisation of the membrane is an early and essential step in differentiation (Bernheim & Bader, 2002), which may also be true for cardiac progenitor cells (van Vliet et al., 2010). In both cell types, undifferentiated cells have a resting membrane potential of ~− 30 mV, which is gradually hyperpolarised when I_{K1} channels become expressed. As a result of this hyperpolarisation, 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 (Darbellay et al., 2010; Liu et al., 1998), but only L-type Ca^{2+} channels were found in human cardiomyocyte progenitor cells (van Vliet et al., 2010).

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 myoblasts (Hinard, Belin, Konig, Bader, & Bernheim, 2008). 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 membrane (Leem et al., 2016). 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.

2.3. 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 summarised 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 (Milstein et al., 2012; Varghese, 2016). This macromolecular complex-mediated trafficking is established by an interplay between the cytoskeleton, motor proteins and cell organelles (Matamoros et al., 2016). 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 channels (Willis, Ponce-Balbuena, & Jalife, 2015). SAP97 and Kir2.1 have also been associated with beta-adrenergic receptor 1 (β-AR1) and Protein Kinase A (PKA) (Vaidyanathan, Taffet, Vikstrom, & Anumonwo, 2010). The interaction between SAP97 and Kir2.1 is likely achieved by a 'Postsynaptic density protein, Drosophila disc large tumour 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 consequences. (Matamoros et al., 2016). Kir2.1 has also been associated with calcium handling, with high levels of intracellular calcium correlating to voltage-dependent I_{K1} block (Zaza, Rocchetti, Brioschi, Cantadori, & Ferroni, 1998) and upregulation of I_{K1} as a consequence of decreased Calmodulin Kinase II (CamKII) activity (Li et al., 2006).

The Kir2.1 channel not only interacts with other channels, but also with different lipids and proteins involved in various cellular processes.

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

Compound	Group	Kir2.1⇓	Kir2.1⇑	Mechanism of action	Cell source
α-1-Syntrophin	Protein		X	Part of macromolecular complex mediating reciprocal modulation of $Na_v1.5$ and Kir2.1	CHO + rat ventricular cardiomyocytes (Matamoros et al., 2016), mouse ventricular myocytes (Gavillet et al., 2006)
AKAP 15 and 79	Plasma membrane- bound proteins	X		Colocalize with Kir2.1 and are involved in PKA signaling	*Xenopus* oocytes + rat ventricular cardiomyocytes (Seyler et al., 2017)
β1-AR	Receptor	X		I_{K1} current decreases as a result of β1-AR activation	Rat ventricular cardiomyocytes (Vaidyanathan et al., 2010)
CaMKII	Kinase		X	Inhibition of CaMKII leads to upregulation of I_{K1}	Mouse ventricular cardiomyocytes (Li et al., 2006)
Caveolin-1	Protein	X		Binds to Kir2.1 channel	HEK293 + mice bone marrow derived macrophages (Han et al., 2016)
Caveolin-3	Protein	X		Protein carrying mutation decreases Kir2.1 surface expression	HEK293 (Vaidyanathan et al., 2013)
Cdo	Cell surface protein		X	Forms complex with Kir2.1 during myogenic differentiation to enhance surface expression of Kir2.1	Myoblast C2C12 (Leem et al., 2016)
Cholesterol	Lipid	X		Influences gating of Kir2.1 channels	CHO (Romanenko et al., 2004), *Xenopus* oocytes (Rosenhouse-Dantsker et al., 2011)
Endothelin-1	Protein	X		PKC-mediated phosphorylation of heteromeric Kir2.1/Kir2.2 channels	Human cardiomyocytes + *Xenopus* oocytes (Kiesecker et al., 2005)
Filamin A	Actin-binding protein		X	Interacts with Kir2.1 to increase number of functional channels	Pig arterial smooth muscle cells (Sampson et al., 2003)
Kir2.2	Ion channel subunit			Forms heterotetramers with Kir2.1	Rabbit ventricular cardiomyocytes (Zobel et al., 2003), HEK293A (Panama et al., 2010)
Kir2.3	Ion channel subunit			Forms heterotetramers with Kir2.1	HEK293 (Muñoz et al., 2007)
microRNA-1	microRNA	X		Decreases KCNJ2 expression	Human cardiomyocytes (healthy + CAD), rat ventricular cardiomyocytes + HEK293 (Yang et al., 2007)
Nav1.5 N-terminal domain	Protein		X	Increases I_{K1} by increasing Kir2.1 expression through reciprocal modulation of Nav1.5	Human atrial and rat cardiomyocytes (Matamoros et al., 2016)
PIKfyve	Protein		X	Increases Kir channel abundance	*Xenopus* oocytes (Munoz et al., 2013)
PI-4-K	Kinase		X	Activates calcium sensing receptors, increases I_{K1} current	HEK293T (Liu et al., 2016)
PIP2	Phospholipid		X	Promotes opening of Kir channel	*Xenopus* oocytes (Xie et al., 2008)
PKA	Protein	X		Inhibits I_{K1} current	*Xenopus* oocytes + rat ventricular cardiomyocytes (Seyler et al., 2017)
PKB	Protein		X	Increases Kir channel abundance	*Xenopus* oocytes (Munoz et al., 2013)
PKC Beta and Kir2.2		X		Decreases I_{K1} current in Kir2.1 & Kir2.2 heterotetramers	*Xenopus* oocytes + rat/mouse ventricular cardiomyocytes (Scherer et al., 2016)
Protein Kinase C	Protein	X		Inhibits I_{K1} current	Human atrial cardiomyocytes + *Xenopus* oocytes (Karle et al., 2002)
SAP97	Protein		X	Associates with Kir2.1	Rat ventricular cardiomyocytes (Vaidyanathan et al., 2010)

One of these is membrane-bound Phosphoinositide 4,5-biphosphate (PIP_2) that increases the open-probability to allow full conductance of I_{K1} (Xie, John, Ribalet, & Weiss, 2008). 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-K (Liu, Chang, Lee, & Shieh, 2016). Cholesterol negatively influences Kir2.1 by regulating the open probability (Romanenko et al., 2004). 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-1 (Han, Rosenhouse-Dantsker, Gnanasambandam, Sachs, & Levitan, 2016). Kir2.1 is further associated with the protein kinase (PK) family, of which PKA and PKC decrease I_{K1} (Karle et al., 2002; Seyler et al., 2017) 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 membrane (Munoz, Almilaji, Setiawan, Föller, & Lang, 2013). The amount of Kir2.1 channels also increases in vascular smooth muscle through interacting with actin-binding protein Filamin A (Sampson, Leyland, & Dart, 2003). Interactions with cardiomyocyte specific proteins have been investigated in zebrafish, where I_{K1} is downregulated after knockout of the desmosomal protein plakophilin-2 (Asimaki et al., 2014). I_{K1} is also negatively regulated by Kir2.1 trafficking. Retrograde Kir2.1 trafficking can be modulated through the clathrin-mediated endocytosis pathway (Varkevisser et al., 2013). Kir2.1 is further regulated on a transcriptional level by microRNA1 (miR-1), which decreases KCNJ2 post-transcriptionally (Yang et al., 2007).[1] Furthermore, Kir2.1 appears be downregulated by miR-26 and miR-101 in atrial fibrillation (Luo et al., 2013).

3. 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 Oct4 (Barruet & Hsiao, 2016). 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 cells (Mummery et al., 2012). All methods use a different approach to spatially organise the cells and yield a different purity of cardiomyocytes.

Next to spatial organisation, 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 (Tirosh-Finkel et al., 2010; Tzahor, 2007). 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 (Calderon, Bardot, & Dubois, 2016, Yang, Pabon, & Murry, 2014). 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 variability (Matsa, 2016) and differences between sexes (Anguera et al., 2012).

3.1. 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, TNNT2 (Puppala et al., 2013), MYL7 (Zhang et al., 2009), MHC α-chain and MHC β-chain and MLC (Gai et al., 2009). Cardiac transcription factors GATA4, GATA6, MEF2 and NKX2.5 have also been reported in iPSC-CMs (Puppala et al., 2013). iPSC-CMs further express genes like KCNH2 (I_{Kr}), KCNQ1 (I_{Ks}), SCN5A (I_{Na}), and CACNA1C & CACNA1D (I_{CaL}) (Karakikes, Ameen, Termglinchan, & Wu, 2015) 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 upstroke (Karakikes et al., 2015). iPSC-CMs also express genes coding for the L-type calcium current, but the sarcoplasmic reticulum contains less SERCA2a which impairs SR-mediated calcium handling (Itzhaki et al., 2011). 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) (Karakikes et al., 2015). As has been mentioned before, iPSC-CMs present high transcriptional levels for HCN4 (I_f) and lack transcription of KCNJ2 (I_{K1}) (Jonsson et al., 2012). 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-CMs (Cordeiro et al., 2013).

3.2. 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 APD prolongation and/or field potential duration (FPD) prolongation with high specificity and sensitivity (Ando et al., 2017; Blinova et al., 2017; Kitaguchi et al., 2015). Drug safety assays using iPSC-CMs work best with drugs blocking the hERG current, which is also most frequently blocked in TdP (Crumb, Vicente, Johannesen, & Strauss, 2016). 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 sodium (Blinova et al., 2017) and may give false predictions for Nav1.5 inhibition (Ando et al., 2017). 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 $I_{Ca,L}$ and I_{K1} block (Paci, Hyttinen, Rodriguez, & Severi, 2015). 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* experiments (Li et al., 2017).

3.3. Making the right comparison: the human foetal 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 foetal cardiomyocyte. However, literature on the electrophysiology of the human foetal 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 utero* (Coltart, Spilker, & Meldrum, 1971; Tuganowski & Cekanski, 1971). 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 foetal cardiomyocytes have a stable and negative resting membrane potential of ~−80 mV. As a result, upstroke velocity of the action

[1] 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 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 (2007)	hESC H1	Long-term culture	At − 90 mV 3.4 ± 1.3 pA/pF	− 87.0 mV
Kim (2010)	hESC-CM	Co-culture with cardiomyocyte spheroids	At − 100 mV − 1.67 ± 0.50 pA/pF	− 85.0 mV
Ma (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 (2012)	hiPSC-CM	Long-term culture	At − 100 mV − 3.49 ± 0.91 pA/pF	− 82.6 mV
Nunes (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 (2016)	hiPSC-CM	Kir2.1 adenovirus	At − 120 mV − 29.8 ± 6.9 pA/pF	− 84.8 mV

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 foetal 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 tube (Fijnvandraat et al., 2003). 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 maturation (Liaw & Zimmermann, 2016).

4. Optimising 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 surfaces (Zhu et al., 2014), applying biochemical stress by electrical stimulation (Zhu et al., 2014), co-cultures with non-cardiomyocytes (Ravenscroft, Pointon, Williams, Cross, & Sidaway, 2016), overexpression, prolonged cell culture (Tzatzalos, Abilez, Shukla, & Wu, 2016), and 3D engineered heart tissues (EHTs) (Feric & Radisic, 2016; Fong et al., 2016). 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 method (Meijer van Putten et al., 2015).

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 model (Wilders, 2006). Injection of I_{K1} into an iPSC-CM will influence AP shape and duration Fig. 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, 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 cells (Dhamoon et al., 2004), the "TNNP" model which is based on I_{K1} in ventricular myocytes in physiological and pathological states (Ten Tusscher, 2003). 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" model (Bett et al., 2013), yielded considerably shorter action potentials, due to the low inward rectification in this model which results in suppression of the plateau.

4.1. Effects of optimisation 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 (summarised 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 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. Most important missing parameter is conductance density (g_{max}, *i.e.* the maximal conductance of the inward component, normalised to membrane capacitance) which is a good measure of the amount of functional I_{K1} channels in the plasma membrane. Calculation of rectification factor (De Boer et al., 2010) 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/pF (Schram, Pourrier, Melnyk, & Nattel, 2002).

Out of the 6 optimisation 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 108 (Sartiani et al., 2007). 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 60 (Kim et al., 2010). 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 121 (Doss et al., 2012). 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 optimisation strategy was applied, with an average of 2.3 pA/pF (Ma et al., 2011). 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 cultures (Nunes et al., 2013). Recently, I_{K1} channels were overexpressed in iPSC-CMs using adenoviral vectors, where I_{K1} current density increased to −30 pA/pF (Vaidyanathan et al., 2016). 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 (Bruegmann et al., 2010; Burton et al., 2015).

4.2. 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 depolarisation of the resting membrane potential from −80.6 ± 1.1 mV to −60.5 ± 2.6 mV (Hoshino, Omatsu-Kanbe, Nakagawa, & Matsuura, 2012). 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 foetal ventricular or atrial cardiomyocytes display strongly depolarised resting membrane potentials (~−40 mV, Mummery et al., 2003) compared to the resting membrane potentials that were recorded *in situ* (~−80 mV, see Section 3.3). Though not tested experimentally, a possible explanation for the depolarisation of primary human foetal 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 7 days in culture (Zhang et al., 2010). 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 h and no further increase or decrease for 6 days (Mitcheson & Levi, 1996), to a gradual decrease of 83% in current density after 4 days in culture (Christe, 1999). In adult canine cardiomyocytes, I_{K1} conductance density decreased approximately 30% between 1 and 7 days in culture, with no significant change in resting membrane potential (de Boer et al., 2006).

Cell culture not only influences ion channel expression of cardiomyocytes, but also the morphology of the cell. Cardiomyocytes become shorter, rounder and lose T-tubules (Louch, Sheehan, & Wolska, 2011), which are invaginations in the adult cardiomyocyte membrane that cooperate with ion channels and receptors to promote excitation-contraction coupling (Ibrahim, Gorelik, Yacoub, & Terracciano, 2011). The reduction of T-tubules in cardiomyocytes has been associated with a reduction of I_{K1} current density (Christe, 1999). Several studies have aimed to restore T-tubules in culture in neonatal rat cardiomyocytes (NRCM). In one study, NRCM were dispersed in a 3D "tissue patch" mimicking the human epicard. After three weeks in culture, the cardiomyocytes showed T-tubulation and co-localisation of L-type calcium channels and ryanodine receptors indicating improved excitation-contraction coupling (Bian, Badie, Iv, & Bursac, 2014). 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 NRCM in a 3D graft. Cardiomyocytes showed increased T-tubulation and improved calcium handling after electrical field stimulation (Godier-Furnémont et al., 2015). Since iPSC-CMs lack T-tubules (Kane, Couch, & Terracciano, 2015), introducing T-tubules in culture might prove to benefit I_{K1} current densities.

5. 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 behaviour 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 stabilise 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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