

Arrhythmia Mechanisms in Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes

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Abstract: Despite major efforts by clinicians and researchers, cardiac arrhythmia remains a leading cause of morbidity and mortality in the world. Experimental work has relied on combining high-throughput strategies with standard molecular and electrophysiological studies, which are, to a great extent, based on the use of animal models. Because this poses major challenges for translation, the progress in the development of novel antiarrhythmic agents and clinical care has been mostly disappointing. Recently, the advent of human induced pluripotent stem cell–derived cardiomyocytes has opened new avenues for both basic cardiac research and drug discovery; now, there is an unlimited source of cardiomyocytes of human origin, both from healthy individuals and patients with cardiac diseases. Understanding arrhythmic mechanisms is one of the main use cases of human induced pluripotent stem cell–derived cardiomyocytes, in addition to pharmacological cardiotoxicity and efficacy testing, in vitro disease modeling, developing patient-specific models and personalized drugs, and regenerative medicine. Here, we review the advances that the human induced pluripotent stem cell–derived-based modeling systems have brought so far regarding the understanding of both arrhythmogenic triggers and substrates, while also briefly speculating about the possibilities in the future.

Key Words: hiPSC-CM, arrhythmia mechanisms, in vitro, in silico
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INTRODUCTION

Despite major efforts by clinicians and researchers, cardiac arrhythmia remains a leading cause of morbidity and mortality in the world.¹ Meanwhile, the introduction of gene expression profiling methods has led to an explosion of data regarding the pathogenesis of a wide variety of heart diseases. Regardless of the great advances in linking the genetic and molecular abnormalities to alterations in electrophysiology, the progress in the development of novel antiarrhythmic

agents and clinical practices approaches has been mostly disappointing during the past few decades.

Crossing the genotype–phenotype gap has relied on combining these high-throughput genetic strategies with standard molecular and electrophysiological studies, which are, to a great extent, based on the use of animal models. When used in functional studies, the murine, small animal, and large animal models each have their own strengths and weakness regarding resources, ethics, methodology, and species-specific physiology. For recent reviews of their use in arrhythmia research, see for example.^{2–4} Whereas, in the human domain, ex vivo organ-level investigations are challenging because ethical restriction limits the access to hearts unused in cardiac transplantation. Cell-level studies, on the other hand, have the practical hindrance that the yield of viable cardiomyocytes (CMs) is typically meager from the tissue samples that are available from certain heart operations. Even more importantly, the access of those surgical samples is limited to only particular regions of the heart and patients with specific cardiac diseases.

The advent of human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) in 2007 has solved one fundamental challenge in basic cardiac research and drug discovery; now, there is an unlimited source of CMs of human origin, both from healthy individuals and patients with cardiac diseases.⁵ The hiPSC-CM discovery opened a plethora of different use cases as follows: tests on drug cardiotoxicity and efficacy, understanding arrhythmic mechanisms, modeling diseases in a dish, developing patient-specific models and personalized drugs, and regenerative medicine. Some use cases are in a more developed state than others (Fig. 1). For example, using hiPSC-CMs for drug cardiac safety assessment was theorized in 2013 within the Comprehensive in vitro Proarrhythmia Assay (CiPA),⁶ as a step to confirm the results of in vitro and in silico tests on drug effects on multiple cardiac ion channels and the cardiac action potential (AP). At the time of this review, the CiPA initiative has already obtained the support of regulatory agencies and pharmaceutical companies, and it is at a very advanced state (cipaproject.org/timelines/). Conversely, other applications are still in their infancy, for example, the development of personalized therapies informed by in-depth analysis of patient-specific hiPSC-CMs and personalized medicine.⁷ Furthermore, the full potential of hiPSC-based applications remains to be harnessed because these cells do not adequately recapitulate (1) morphological and ultrastructural, (2) electrophysiological, (3) contractile, and (4)

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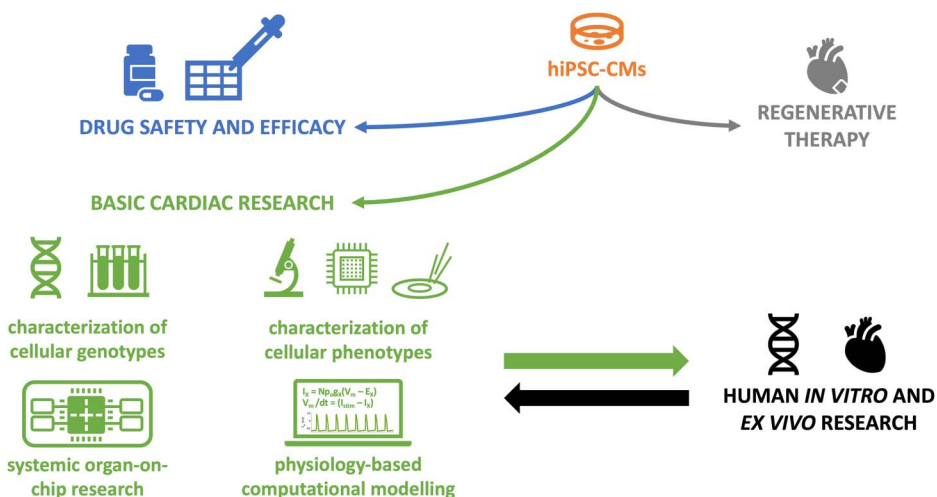


FIGURE 1. Scope of the review. Although arrhythmogenic properties of hiPSC-CMs are very relevant in all 3 principal application fields (basic research, drug screening, and regenerative therapy), we focus on the first one and on the second one to a lesser extent. Also, we link the hiPSC-based findings to the human/patient context whenever possible.

metabolic properties of native adult human CMs. Efforts aimed at solving these challenges have been recently reviewed by others.^{8–12}

Here, we review the advances that the hiPSC-based modeling systems have brought so far regarding the understanding of both arrhythmogenic triggers and substrates. First, we assess how different mechanisms of arrhythmogenesis that relate to abnormal impulse formation and propagation have been captured. Then, we explore to what extent can hiPSC-based approaches recapitulate the different atrial and ventricular arrhythmogenic phenotypes. The usability of hiPSC-CMs in investigating drug-induced arrhythmias and sex-dependent arrhythmic characteristics are discussed in the following chapters. Finally, we provide some conclusions and speculate about future directions.

ARRHYTHMOGENESIS

One of the aforementioned benefits of hiPSC-CMs is the availability of a pool of in vitro human cell models to reproduce the occurrence of arrhythmias under different conditions, and to understand better the underlying mechanisms,¹³ as well as developing pharmacological strategies to reduce them. Examples of use cases are the presence of mutations altering specific membrane channels (channelopathies)^{14–20} or other cell functions,^{21–25} or when administering drugs.²⁶ For all these use cases, the clear advantage of hiPSC-CMs is that they are from human source. A potential disadvantage is that they are relatively recent in vitro models, and a better understanding of the actual similarity to native human CMs in terms of arrhythmic event development is needed.²⁷

A well-known scheme to classify mechanisms of cardiac arrhythmias^{28,29} identifies 2 macrofamilies, separating nonreentrant and reentrant activities: abnormal impulse formation and improper impulse conduction. In this review, we follow this scheme (Fig. 2), reporting abnormal impulse formation and improper impulse conduction for hiPSC-CMs

and expand the mechanisms adding spatial dispersion of repolarization and myocardial heterogeneity.

Abnormal Impulse Formation

Abnormal impulse formation is traditionally split into 2 subcategories: triggered activity and automaticity.³⁰ At the cell level, triggered activity refers to electromechanical events, that is, anticipated APs and the contraction of the CMs, generated by an early or delayed afterdepolarization (DAD), and not by an external pacing stimulus (eg, an external current during patch-clamp experiments or the nodal pacemaking in the heart). The triggered activity at the cell level can initiate tachyarrhythmias at the whole organ level. Automaticity refers to the property of CMs to generate spontaneous APs. The automaticity of the sinoatrial node sets the pacing of the heart. Therefore, automaticity per se is a physiological phenomenon. However, abnormal automaticity can lead to too slow (bradycardia) or too fast (tachycardia) rhythm or even trigger arrhythmias, for example, atrial fibrillation (AF) as a consequence of malfunctioning atrioventricular node. In the next sections, we summarize how afterdepolarizations, triggered activity, and automaticity manifest in hiPSC-CMs and the underlying mechanisms.

Triggered Activity

Early afterdepolarizations (EADs) are likely the most known proarrhythmic marker at the cellular level, and they consist of a nonphysiological depolarization of the CM membrane potential during phase 2 or 3 of the AP. The primary mechanism triggering an EAD is that the inward flux of positive charges into the cytosol is higher than the outward flux, during those AP phases where the main actors are physiologically the repolarizing rapid and slow delayed rectifying K⁺ currents (I_{Kr} and I_{Ks}), and the inward Na⁺ and Ca²⁺ currents should be inactivated. The main risk connected with EAD development is that a secondary/triggered AP could follow the primary AP, if the afterdepolarization is strong enough to reach the threshold membrane potential and the fast Na⁺ current (I_{Na}) has recovered from preceding inactivation.

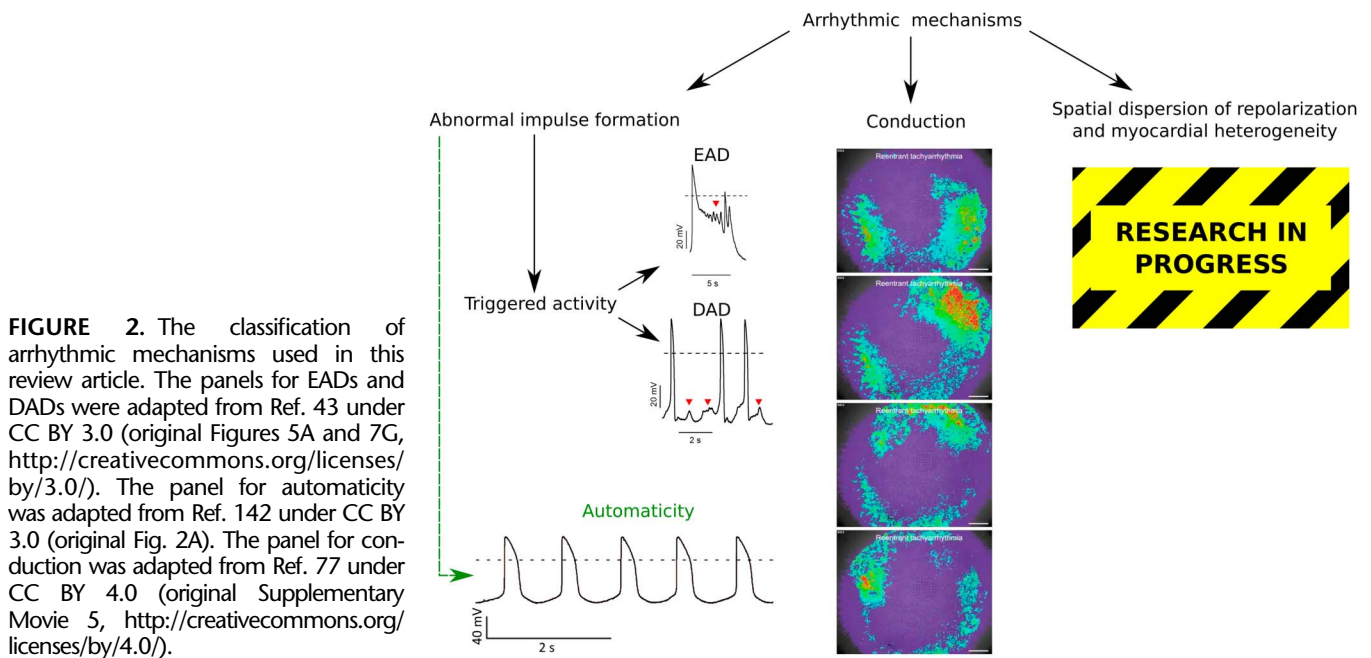


FIGURE 2. The classification of arrhythmic mechanisms used in this review article. The panels for EADs and DADs were adapted from Ref. 43 under CC BY 3.0 (original Figures 5A and 7G, <http://creativecommons.org/licenses/by/3.0/>). The panel for automaticity was adapted from Ref. 142 under CC BY 3.0 (original Fig. 2A). The panel for conduction was adapted from Ref. 77 under CC BY 4.0 (original Supplementary Movie 5, <http://creativecommons.org/licenses/by/4.0/>).

The most common scenario for the development of EADs is the prolongation of the AP: the repolarization is slowed down, for example, by a channelopathy or by drugs blocking I_{Kr} , I_{Ks} , or both. In these conditions, the L-type Ca^{2+} current (I_{CaL}) can reactivate, thus allowing an inward Ca^{2+} flux and causing depolarization.³¹ The second possible trigger for EADs in conditions of prolonged AP is spontaneous releases of Ca^{2+} from the sarcoplasmic reticulum (SR), for example, in case of overload.³² In a similar fashion to the development of DADs, but before the full-membrane repolarization, Ca^{2+} spontaneously released from the SR activates the Na^+/Ca^{2+} exchanger (I_{NCX}) that expels one Ca^{2+} ion out of the cell and brings in 3 Na^+ ions. This net inward flux depolarizes the membrane potential during its repolarization.^{33,34}

A second scenario, involving not prolonged AP but shortened, was described by Burashnikov et al as late phase 3 EADs in canine preparations.^{35,36} In conditions of (1) very short APs (induced by the parasympathetic neurotransmitter acetylcholine) and (2) a normal release from SR (enabled by the short AP), the cytosolic Ca^{2+} concentration could remain elevated when the membrane potential is more negative than the I_{NCX} equilibrium potential. These 2 conditions are sufficient to activate I_{NCX} in an inward mode, which depolarizes the membrane potential,²⁹ thus interrupting the late phase of repolarization. Phase 3 EADs were also observed as consequence of nonequilibrium reactivation of I_{Na} in murine ventricular cells (in vitro and in silico)³⁷ and in human atrial cells (in silico).³⁸ Despite the 2 cell types are different, they share certain common features: (1) the repolarization trajectory is triangular and with short and negative plateau and (2) the late repolarization phase is strongly dependent on the sarcoplasmic Ca^{2+} release and inward I_{NCX} . Simulations on human atrial cardiomyocytes³⁸ showed that the negative AP plateau accelerates I_{Na} recovery from inactivation, enabling I_{Na}

reactivation when the AP is prolonged by increased Ca^{2+} extrusion by I_{NCX} , triggering phase 3 EADs. Experimental evidence was observed in vitro on ventricular murine cells, where phase 3 EADs were not suppressed by the administration of ranolazine (selective current blocker for the late Na^+ current, I_{NaL}), but of terodotoxin (mainly targeting the fast I_{Na} and only to a lesser extent I_{NaL}).

Developing EADs in the same conditions when they are triggered in native human preparations is a property that hiPSC-CMs must show to be considered a reliable in vitro CM model. EADs developed both in control and mutant hiPSC-CMs. For example, Ma et al³⁹ reported EADs in control hiPSC-CMs as a consequence of a strong block of I_{Kr} (100 nM E4031) and bradycardia-like conditions (pacing rate 0.5 Hz) but not at 1 Hz pacing. In addition to EAD rate dependence, Ma et al showed a dependency between EAD take-off potential and peak voltage, nearly identical to what was observed in native canine CMs. During the last decade, a plethora of studies showed hiPSC-CM vulnerability to EADs in the presence of mutations in genes encoding ion channel, as reviewed by Pourrier et al.¹³ The most common channelopathies studied reproduced in hiPSC-CMs belong to the long QT (LQT) family (see section Ventricular). EADs were observed in hiPSC-CMs carrying mutations for LQT1 (I_{Ks} loss of function),^{14–16} LQT2 (I_{Kr} loss of function),^{15–20} and LQT3⁴⁰ (gain of function of I_{NaL}). It is also interesting that mutant hiPSC-CMs were more prone than controls to EADs, after in vitro and in silico I_{Kr} blockers (E4031, cisapride, and quinidine) or isoproterenol administration^{14,16,17,41} or even without drugs¹⁹ and in case of a slow pacing rate (0.2 Hz).⁴⁰ Also, late phase 3 EADs have been reproduced in hiPSC-CM expressing mutations for catecholaminergic polymorphic ventricular tachycardia (CPVT) (Fig. 4 in Ref. 25).

DADs consist of unphysiological depolarizations once the membrane potential is fully repolarized. Some of the considerations we proposed for EADs stand also for DADs. First, the primary mechanism is again an inward flux of positive charges that destabilizes the normal time-course of the AP, happening in phase 4 for DADs and not in phase 2 or 3 as for EADs.²⁹ Second, the spontaneous release of Ca²⁺ from SR that activates the inward I_{NCX} is the trigger for DADs, similar to EADs. Third, DADs could trigger AP if their peak voltage is high enough. Differently from EADs, which are usually developed in bradycardia-like conditions, DADs usually develop at high-pacing frequencies, which do not allow enough time for a normal physiological Ca²⁺ release, thus causing SR overload.²⁸ Other conditions creating a substrate for DADs are an elevated extracellular⁴² and intracellular⁴³ Ca²⁺ concentration, hypertrophic cardiomyopathy (HCM),²⁸ or mutations in the RyR2 gene that encode for the Ca²⁺ release channels.⁴⁴

DADs have been successfully modeled in disease-specific hiPSC-CM¹³ (see section Ventricular). Furthermore, CPVT mutations have been shown to induce DADs in hiPSC-CMs, which are exacerbated by adrenergic stimulation (eg, isoproterenol or forskolin).^{20,45–47} Also, in the presence of mutations inducing HCM, hiPSC-CMs developed DADs reasonably because of the elevation of the cytosolic free Ca²⁺.^{21–23} The aforementioned mechanisms underlying DAD formation were confirmed by means of *in silico* hiPSC-CM models.^{48,49} Furthermore, Koivumäki et al⁵⁰ replicated CPVT-like membrane potential traces, showing that hiPSC-CMs are more prone to these arrhythmogenic events than their adult counterparts.

Automaticity

Automaticity is one key feature of hiPSC-CMs, that is, they can trigger APs and, consequently, contract without an external stimulus. hiPSC-CM automaticity is a double-edged sword. On the one hand, it is a clear macro-marker of hiPSCs differentiation toward the cardiac phenotype.³⁹ On the other hand, it is a sign of immaturity as CMs,⁵¹ as detailed in the following.

hiPSC-CMs and their pacemaking activity have been studied for about a decade,⁵² and 2 mechanisms have been identified as follows: membrane clock (also known as voltage clock or M clock) and Ca²⁺ clock. A common element between membrane clock and Ca²⁺ clock in hiPSC-CMs is the weak inward rectifying K⁺ current (I_{K1}). In adult CMs, I_{K1} is the principal actor in the AP resting phase, playing a fundamental role in stabilizing the resting membrane potential (RMP)⁵³ around $-80/-90$ mV.⁵⁴ Conversely, RMP recorded in spontaneously beating hiPSC-CMs is strongly depolarized: -76 mV,³⁹ -64 mV,⁵⁵ -61 mV,⁵⁶ -63 mV,²⁰ -68 mV,²⁴ or -59 mV.⁵⁷ Only a few groups succeeded in recording I_{K1} in hiPSC-CMs and reporting I_{K1} densities: -2.3 pA/pF at -123 mV,³⁹ ~ -1.1 pA/pF,⁵⁷ or -5.1 pA/pF.⁵⁸ These values are significantly smaller than I_{K1} in adult human CMs (~ 11.5 pA/pF,⁵⁹). In the more recent study by Horváth et al,⁶⁰ experiments indicated that I_{K1} could strongly depend on the hiPSC-CM line and RMP on the measuring technique. hiPSC-CMs from a proprietary line cultured as monolayers or

3D engineered heart tissues (EHTs) exhibited an I_{K1} density similar to left ventricular or right atrial CMs (32.7 and 14.1 vs. 40.5 and 14.0 pA/pF). Conversely, I_{K1} was 2-fold smaller in EHTs built with commercially available hiPSC-CMs (iCell). In agreement with the other sources reported in this paragraph, Horváth et al measured depolarized RMP in isolated hiPSC-CMs by patch-clamp (-59.66 mV). Nevertheless, measurements from hiPSC-CMs in EHTs by means of sharp microelectrodes provided RMP values similar to adult CMs (-74.58 vs. -75.87 mV). Thus, the authors concluded that hiPSC-CM I_{K1} is not necessarily lower than the adult one, and technical issues may underlie the depolarized RMP in hiPSC-CMs. Of note, RMPs measured on adult ventricular and atrial CMs did not depend so dramatically on the measuring technique, indicating that the small size of hiPSC-CMs might play a role in technical challenges of RMP measurements.

Membrane clock is based on (1) the hyperpolarization-activated, cyclic nucleotide-gated (HCN) current or funny current (I_f) that acts as the pacemaking trigger,^{61–63} combined with (2) a very weak I_{K1}. I_f is a nonselective depolarizing current deriving from the influx of Na⁺ and efflux of K⁺ ions through the HCN channels, activated at hyperpolarized voltages of the cell membrane (-45 to $-60/-70$ mV).^{57,64} Many studies in the literature support the idea of I_f as pacemaking current in hiPSC-CMs.^{51,52,65–68} Given the inability of I_{K1} in stabilizing the membrane potential, I_f is enough to promote depolarization during the diastolic phase (or phase 4) of the AP, named diastolic depolarization, up to the opening threshold for I_{Na} and I_{CaL} triggering the upstroke. I_f typical values in hiPSC-CMs are -4.1 pA/pF at -120 mV,³⁹ ~ -5 pA/pF at -125 mV,⁶⁶ and ~ -4 pA/pF at -125 mV.⁵² Lee et al⁵⁸ reported a smaller value of zatebradine-sensitive current (-0.9 pA/pF) that could be an underestimation of I_f since zatebradine blocks about 50% of I_f.⁵² Experiments testing the effect of I_f block by ivabradine showed a strong reduction of the spontaneous rate up to quiescence.^{65,68}

Conversely, Ca²⁺ clock does not rely on I_f as pacemaker current but on a complex interplay between the SR and the Na⁺/Ca²⁺ exchanger. To date, only one study demonstrated *in vitro* this mechanism in hiPSC-CMs.⁵⁷ Ca²⁺ clock works similarly to the mechanism underlying the development of DADs (see section Triggered Activity): spontaneous Ca²⁺ release from the SR elevates the cytosolic Ca²⁺ concentration, which activates I_{NCX} in inward mode, that is, one Ca²⁺ ion is transported to the extracellular environment by transporting 3 Na⁺ ions in the CM cytosol. The resulting influx of positive charges, combined with a weak I_{K1} as also observed by Kim et al,⁵⁷ is enough to trigger a diastolic depolarization as I_f does in the membrane clock model. Shortly, to support Ca²⁺ clock in hiPSC-CM, Kim et al assessed the actual expression of HCN genes in their hiPSC-CMs and then administered ivabradine, a selective I_f blocker, up to 9 μM. Ivabradine failed to alter the rate of spontaneous Ca²⁺ transients significantly. Furthermore, voltage-clamp recordings found a negligible I_f; still, their hiPSC-CMs showed an automatic rate of about 1 Hz. Conversely, I_{NCX} inhibition by SEA0400 and ryanodine receptor stabilization by tetracaine successfully reduced the spontaneous rate up to

quiescence. Interestingly, SEA0400 induced controversial responses in hiPSC-CM-based EHT.⁶⁸ Here, the authors showed that some EHTs increased their spontaneous rate, whereas others reduced it, and some ceased beating at high concentrations. This is not surprising because I_{NCX} is fundamental in maintaining the homeostatic intracellular Na^+ and Ca^{2+} concentrations. Although it is reasonable that the block of the pre-upstroke inward component of I_{NCX} reduces the spontaneous rate and even block automaticity, the effects on the cytosolic ion concentrations could drive the cell to an unphysiological state and cannot be neglected. hiPSC-CM automaticity was investigated also by means of *in silico* models. Koivumäki et al⁵⁰ developed an *in silico* model replicating the Ca^{2+} clock mechanism highlighted *in vitro* by Kim et al⁵⁷ and showed I_f negligible role in sustaining spontaneous APs. Conversely, Paci et al⁴⁹ developed a model where both Ca^{2+} clock (mediated by Ca^{2+} and I_{NCX}) and voltage clock (mediated by I_f) coexist. Given the high variability showed by hiPSC-CMs, it is reasonable to hypothesize that both mechanisms could exist physiologically and one or the other could be more predominant in specific cell cultures. This is the reason why many computational studies, especially dealing with hiPSC-CM modeling, do not focus on developing a single model anymore, but populations of models, to capture also the experimental variability in their simulations.^{41,49,50,69,70}

It is interesting noting that the debate about hiPSC-CM automaticity mirrors a similar discussion that happened more than a decade ago for adult pacemaking cells in the heart. Also for adult nodal cells, both membrane clock and Ca^{2+} clock had their supporters, among whom the most eminent are DiFrancesco et al⁶¹ and Maltsev and Lakatta,⁷¹ respectively. In 2009, Joung et al⁷² concluded that membrane clock and Ca^{2+} clock coexist and synergically contribute to automaticity in sinoatrial nodal cells. More recently, in 2015, Yaniv et al,⁷³ developed the concept of coupled clock, supported by experimental evidence and *in silico* simulations, and integrating the 2 previous oscillation mechanisms. However, a detailed analysis of the coupled clock is out of the scope of this review.

hiPSC-CM automaticity is not only a macromarker of differentiation toward CMs but also of immaturity, together with cell morphology and disorganization of the contractile apparatus⁹ and diverse expression of ion currents.⁷⁴ This was highlighted more than a decade ago about CMs derived from human embryonic stem cells (hESCs).⁷⁵ Among the developmental changes reported by Sartiani et al from early (15–40 days) to late (50–110 days) hESC-CMs, they observed a significant reduction of 2 isoforms of the HCN channels (1 and 4) and a slower I_f activation rate. Furthermore, late hESC-CMs showed a significantly higher I_{K1} density than early (3.4 vs. 0.6 at -90 mV). Among the developmental changes reported by Pioner et al,⁵¹ a significant decrease of the spontaneous rate was observed at 3 stages of maturation (20, 30, and 60 days), together with a prolongation of the AP with a more marked plateau, a more negative RMP, and an increased response to external electrical pacing. In short, hiPSC-CMs were moving toward a more adult human phenotype during their maturation.

Automaticity also represents a challenge when using hiPSC-CMs for drug tests,⁶⁴ in particular cardiac safety

assessment, as promoted by the CiPA initiative.^{6,76} Goversen et al⁶⁴ reported hiPSC-CM automaticity, and particularly the reduced I_{K1} density, as contributors to proarrhythmic traits, that could bias the response to drugs. The need for establishing a stronger I_{K1} in hiPSC-CMs to reduce or even suppress automaticity is a well-known challenge^{27,64} and crucial for achieving even more reliable human-based CM models for drug tests.

Conduction

Contrary to the ion current and AP characterization, the conduction studies require more complexity of the hiPSC-CM cultures. Regardless of the emerging 3D culturing techniques,⁷⁷ most of the disease modeling and drug toxicity testing are performed with single-cell stage hiPSC-CMs. However, the studies of complex cardiac conduction and arrhythmia mechanisms require a more physiological model system where hiPSC-CMs are electrically and mechanically connected. Therefore, for example hiPSC-CMs as monolayers have been used to study conduction and signal propagation.⁷⁸

For the electrical signal to propagate effectively in the cardiac tissue, the CMs have to be connected tightly with each other by gap junctions. Gap junctions are intercellular channels formed by proteins of the connexin (Cx) family and allow passage of small ions and molecules between neighboring cells.⁷⁹ Gap junctions are located in the intercalated discs, together with desmosomes and adherent junctions. These structures have been first observed already in 1866 by Eberth and are considered to be responsible for the electrical and mechanical coupling of CMs.^{80,81} There are 15 Cx subtypes defined by their molecular weight, each with varying channel properties and gating mechanisms, and Cx 30.2, 40, 43, and 45 have been found to be expressed in mammalian cardiac tissue.^{82–84}

Another key factor in CM excitability and initiation of the AP is the fast voltage-gated Na^+ channel ($Na_v1.5$). According to the recent studies, there might be a mechanistic link between Cx43 and $Na_v1.5$ in addition to their similar location at the intercalated discs.^{85–87} This structural assembly may explain also the functional coupling of cell excitability and impulse propagation governed by $Na_v1.5$ and gap junctions, respectively.⁸⁵ $Na_v1.5$ and Cx43 play a significant role in signal propagation and excitation, and the disturbances in their function or expression levels may lead to slowed and dispersed conduction, which makes the heart susceptible to develop ventricular arrhythmias.⁸⁸

The immature phenotype hinders the use of hiPSC-CMs as models for cardiac excitability as well connectivity. It has been shown that hiPSC-CM-based tissue constructs express reduced levels of Cx43 and gap junction formation compared with the human counterparts, resulting in weak intercellular coupling, low cellular excitability, and low conduction velocity.⁸⁹ Despite these limitations, hiPSC-CMs have been used in studying cardiac conductivity and arrhythmogenesis. Mehta and coworkers assessed the conduction velocity of the beating hiPSC-CM clusters plated on a microelectrode array (MEA). The conduction velocity of hiPSC-CMs ranged from 1.5 to 2.5 cm/s.⁹⁰ Zhang and coworkers used voltage-sensitive dyes together with optical mapping to quantify AP propagation in

hiPSC-CM monolayers. According to the activation maps, the electrical impulse propagated with conduction velocities ranging from 13 to 18 cm/s.⁹¹ With the more complex hiPSC-CM constructs, such as ventricular Biowires, hiPSC-CMs have been used to form thin, cylindrical tissues suspended between 2 wires. This platform enables simultaneous quantification of force and Ca²⁺ transients as well as electrical long-term stimulation. With these constructs, conduction velocities reached 31.8 ± 7.9 cm/s, near the range of adult myocardium.⁹² Recently, Goldfracht et al⁹³ reported a similar type of ventricular and atrial specific EHT constructs to have conduction velocities of 21.4 ± 4.4 cm/s and 4.1 ± 0.2 cm/s, respectively.

Cardiac differentiation methods produce heterogeneous population of cells, containing all cardiac subtypes; ventricular, atrial, and nodal-like cells.^{94–97} In addition to CMs, the cell population contains other cell types. Because of these issues, the nature or quantity of cells in the construct cannot be fully controlled although the hiPSC-CMs would be enriched after differentiation. The aforementioned uncontrolled heterogeneity, conduction blocks and slow AP conduction in the constructs can cause disturbances not only in the basic function but also promote conduction alterations and arrhythmogenesis. For example, conduction blocks can cause the formation of slow conducting re-entry circuits, which is one mechanism responsible for cardiac arrhythmias, as reviewed.^{82,98} A brilliant *in silico* study started tackling these problems, by developing a multiscale framework (cell, tissue, and whole organ levels) to predict how cell therapy, including the virtual injection of hiPSC-CMs, could affect postmyocardial infarcted hearts.⁹⁹ In particular, their simulations showed how the injection of hiPSC-CMs or the use of cell grafts could trigger arrhythmogenic phenomena like ectopic propagation of the hiPSC-CM automaticity or reentries.

It is important to keep in mind that native myocardium contains not only CMs but other types of cells as well. Therefore, the multicellularity of the hiPSC-CM based constructs is a desired phenomenon; however, the cell types and content should be proper. Kawatou et al⁷⁷ increased the cell heterogeneity and used CMs and nonmyocytes in multi-sheet construct with varying percentages for modeling torsades de pointes (TdP) arrhythmia. By increasing the construct heterogeneity and dimensions from 2D to 3D, they were able to induce pharmaceutically spiral wave re-entries. On the contrary, reentry events have been also monitored in differentiated 2D hiPSC-CM monolayer cultures without any CM enrichment procedures.¹⁰⁰ According to the result of these studies, it can be concluded that hiPSC-CM-based models have great potential to be used as a platform to study pathological mechanisms of conduction disorders and reentry. However, more optimization and improvement are still needed before the modeling is reproducible and reliable.

Spatial Dispersion of Repolarization and Myocardial Heterogeneity

In addition to the different subtypes of CMs in the atria and ventricles, CMs have also varying electrophysiological, mechanical, and metabolic characteristics depending on the

location in the heart, as reviewed earlier.¹⁰¹ For example, depending on the transmural location in the human ventricular wall, there are 3 electrophysiologically different cell types present: epicardial, endocardial, and M cells.¹⁰² The varying electrophysiological characteristics of ventricular wall layers have an effect on the dispersion of repolarization in the ventricle, and the disturbances in dispersion of repolarization have been reported to contribute to the development of cardiac arrhythmias, such as TdP.^{103,104}

The number of studies describing the M cells, transmural dispersion of repolarization (TDR), and the link to arrhythmogenesis in human is relatively low, and to the best of our knowledge, the studies of TDR by hiPSC-based models are lacking. Assessing the arrhythmogenesis caused by TDR requires a complex cell construct, which is not obtainable with current methods. Protocols for producing hiPSC-CMs of atrial and ventricular subtypes do exist.^{93,105–107} However, methods for differentiation and characterization of endocardial, epicardial, and M cells would be required for creating platforms to study TDR with hiPSC-CMs. In addition, the non-CMs present in the native myocardium should be included to the construct for reliable modeling of TDR and the effects of heterogeneity to arrhythmogenesis in human heart.

Could *in silico* modeling offer a “quick fix solution” to these challenges? Most likely, yes. We and others have already demonstrated in our previous work that biophysic-based computational modeling can extrapolate impacts of drugs^{108–110} and disease-related alterations⁵⁰ from hiPSC-CMs to native human adult ventricular CMs (hV-CMs). Using the same principle, TDR can be simulated by implementing the *in vitro* hiPSC-CM findings of disease-related alterations to *in silico* tissue geometry to extrapolate their impact to a higher dimension (2D) or even further to the patient context (3D).

MODELLING OF DIFFERENT ARRHYTHMOGENIC PHENOTYPES

A summary of the publications referenced in this section is reported in Table 1.

TABLE 1. Summary of the Publication References for Each of the Arrhythmogenic Phenotypes Described in this Review

Arrhythmogenic Phenotype	References
Atrial fibrillation	114–116
Ischemic ventricular tachycardia	118,119
Hypertrophic cardiomyopathy	21,23,121,123–127
Dilated cardiomyopathy	131–136
Long QT 1	14,55,141–143
Long QT 2	17,19,20,144–147
Long QT 3	40,56,147–150
Long QT 7	152
Long QT 8	153
Brugada syndrome	56,149,157,158
Catecholaminergic polymorphic ventricular tachycardia	24,25,45–47,166–172
Drug-induced arrhythmias	26,74,177–180

Atrial

Although there is already a good selection of articles demonstrating and establishing novel hiPSC-based platforms for studying atria-specific electrophysiology *in vitro*^{92,93,111–113} only a handful of them have actually investigated arrhythmogenic mechanisms of AF.

Marczenke et al¹¹⁴ were the first exploiting the potential of hiPSC-CMs in this context, linking a mutation in the KCNA5 gene, which encodes K_v1.5 channels, mediating the ultrarapid delayed rectifier K⁺ current (I_{Kur}), to a putative trigger mechanism in familial AF. The authors showed that a cholinergic agonist (carbachol, activator of the acetylcholine-sensitive K⁺ current, I_{KACH}) promoted chaotic spontaneous beating in atrial CMs that were K_v1.5 deficient. Whereas, those kinds of abnormalities were not seen in isogenic control CMs. Furthermore, the KCNA5 knockout CMs were shown to be more prone to develop EAD-like triggering events; a putative mechanism for the macroscopic beating irregularities. Nikolova-Krstevski and colleagues¹¹⁵ were also one of the early adopters of the approach. They studied the role of endocardial endothelium in atrial mechano-electrical feedback, showing that (1) in acute stretch, increased transient receptor potential channel activity might protect against myocardial Ca²⁺ overload, whereas, (2) during persistent stretch, reduced activity might be arrhythmogenic. More recently, Benzoni et al¹¹⁶ investigated a familial form of AF and identified a gain of function of I_f and I_{CaL} in hiPSC-CM obtained from 2 patients. Functional analysis showed that the AF-affected CMs displayed higher amplitude DADs and more ectopic beats under stressful conditions (isoproterenol stimulation) compared with healthy control CMs.

There are plenty of studies demonstrating, for example, that (1) hiPSC-CM lines retain AF-related mutations in genes encoding transcription factors and transmembrane ion channels, as well as, (2) hiPSC-CM constructs can replicate relevant geometrical aspects of the human atria. Clearly, there is great potential in hiPSC-based approaches for investigating arrhythmogenic factors of AF.

Importantly, they enable discrimination of primary and secondary/decompensatory causes of AF. Thus, much needed data on the distinct impact of arrhythmogenic alterations can be obtained, when the long-term functional and structural remodeling has not yet taken place. All this is, of course, dependent on both the development of differentiation protocols for deriving true atrial hiPSC-CMs and standardization of biomarkers for verifying the chamber-specific CM phenotype. There is a dire need for these novel *in vitro* methods, as access to native human atrial tissue and CMs is in practice limited to only left and right atrial appendages samples, whose properties differ substantially from those of the working atrial myocardium.

Ventricular

Ischemic Ventricular Tachycardia

Ischemic heart disease and acute myocardial infarction are leading causes of death worldwide. Myocardial infarction often triggers arrhythmia when the human heart is subjected to acute hypoxia during coronary occlusion or when the

oxygen demand of the heart exceeds its workload.¹¹⁷ Although several studies have demonstrated hiPSC-disease modeling regarding inherited ventricular tachycardia, not many have investigated arrhythmogenic mechanisms of acquired ischemic ventricular tachycardia. Wei et al¹¹⁸ evaluated the contractility of hiPSC-CMs and showed that it was reduced during hypoxia exposure compared with the control conditions. Fernandez-Moralez et al¹¹⁹ studied the effects of acute hypoxia and acidosis on hiPSC-CMs and found the connection to the inactivation kinetics of L-type Ca²⁺ channels, showing that the older cells, with slowly inactivating I_{CaL}, were more sensitive to hypoxia but not to acidosis as compared with younger cells.

HCM

HCM is one of the most common genetic cardiac diseases, and it is inherited in an autosomal dominant pattern. Most of the mutations are found either in the β -myosin heavy chain (MYH7) or in the myosin-binding protein C (MYBPC3) genes.¹²⁰ In HCM, the myocyte disorganization and hypertrophy might lead to reentry and thus increase arrhythmia susceptibility. Indeed, several HCM hiPSC-CM studies have reported cellular enlargement, disorganized sarcomeres, multinucleation, or myofibrillar disarray.^{21,23,121} Arrhythmias in HCM can also be caused by secondary effects, such as CM hypertrophy increasing CM automaticity,¹²² but to our understanding, no HCM hiPSC-CM studies have investigated this. One arrhythmia mechanism in HCM is altered cytosolic cellular Ca²⁺ homeostasis and disturbed Ca²⁺ handling, causing triggered activity through depolarization abnormalities.¹²² This mechanism has been evident in several HCM hiPSC-CM studies showing mostly DADs, prolonged AP duration (APD), and/or Ca²⁺ handling abnormalities.^{21,23,121,123,124} Also, EADs and ventricular tachycardia have been reported.¹²⁵ Wu et al¹²⁶ showed that cytosolic diastolic Ca²⁺ overload, and therefore disturbed Ca²⁺ signaling, is an important cellular pathological mechanism of diastolic dysfunctions, which are common with HCM patients. Reduced ATP/ADP ratio and mitochondrial membrane potential have been shown to elevate the intracellular Ca²⁺ concentration, which has been associated with numerous HCM-specific electrophysiological abnormalities.¹²⁷ Also, increased myofilament Ca²⁺ sensitivity and impaired relaxation,^{123,124} as well as enhanced I_{CaL} has been reported with HCM hiPSC-CMs.¹²⁴

Dilated Cardiomyopathy (DCM)

DCM is characterized by weakening of the heart muscle due to a progressive loss of functional CMs, an increase in the size of the ventricular chambers, reduced cardiac output, and arrhythmias.¹²⁸ Numerous mutations affecting the sarcomere and the cytoskeleton have been identified in DCM in more than 30 different genes.¹²⁹ Arrhythmia mechanisms of DCM are poorly understood, but it is believed that the structural abnormalities in myocardium influence the shape and amplitude of the intracellular Ca²⁺ transient, leading to afterdepolarizations.¹³⁰ Also, decreased transient outward K⁺ current (I_{to}), and I_{K1} are believed to contribute to the AP prolongation at the cellular level.¹³ DCM-specific hiPSC-CMs have contraction

and Ca^{2+} handling abnormalities,^{131–133} as well as elevated cytosolic Ca^{2+} levels and increased arrhythmogenic sensitivity to β -adrenergic stimulation.^{133,134} Besides, decreased response to isoproterenol has been reported.^{131,135,136} Slower spontaneous beating rate of DCM hiPSC-CMs has been illustrated,^{133,135} and MEA recordings have revealed increased field potential duration (FPD) in DCM-CM aggregates compared with controls as well as premature beats and VT-type arrhythmias.¹³³ When published DCM hiPSC-CM studies have been examined, the most consistent abnormality has been lower peak force development compared with the respective control, which correlates with the clinical presentation of the disease.¹³⁷

LQT Syndrome

LQT syndrome is an inherited cardiac disorder characterized by a marked prolongation of the QT interval with an increased risk of developing a polymorphic ventricular tachycardia known as TdP and sudden cardiac death.¹³ LQT is currently associated with hundreds of mutations in 17 different genes encoding ion channels and ion channel-modulating proteins. However, in most of the LQT cases, the disease is caused by loss-of-function mutations in genes *KCNQ1* (LQT1) and in the human ether-a-go-go-related gene, *hERG*, (LQT2) or by gain-of-function mutations in *SCN5A* (LQT3),¹³⁸ as briefly introduced in section triggered activity. At the cellular level, LQT mutations result in prolongation of the repolarization phase of the AP, because of a decrease in the net outward current secondary to an increase in I_{Ca} , late I_{Na} , and/or a reduction in I_{Kr} , I_{Ks} , or I_{K1} .¹³ Several studies about LQTS hiPSC-CM modeling have been published during the last decade, mainly focusing on LQTS1, LQTS2, and LQTS3. Common in vitro phenotypes across all LQTS subtypes include prolonged AP, field potential, Ca^{2+} transient and contraction durations, as well as arrhythmogenic events such as DADs and early EADs in baseline conditions or after drug treatment.¹³⁹ These phenotypes reflect well the electrophysiological features of the disorder observed in patients.¹⁴⁰

LQT1 hiPSC studies have corroborated the clinical finding of AP prolongation in patient-specific hiPSC-CMs.^{14,55} The first study with hiPSC-CMs from an LQT1 patient revealed AP prolongation and decreased I_{Ks} in which β -adrenergic stimulation exacerbated AP prolongation and caused EADs, but pretreatment with propranolol diminished the effects.⁵⁵ In another LQT1 study, the I_{Ks} blocker chromanol 293B significantly prolonged FPD in control cells but not in LQT1 hiPSC-CMs, suggesting I_{Ks} dysfunction. I_{Kr} blocker E4031 prolonged FPD and induced frequent severe arrhythmia in LQT1 hiPSC-CMs, indicating reduced repolarization reserve.¹⁴ In Ma et al's¹⁴¹ study, LQT1 CMs demonstrated reduced I_{Ks} current, decreased activation of I_{Ks} , and prolonged APD. Furthermore, ML277, a selective activator of I_{Ks} , reversed the electrophysiological phenotype of the LQT1 CMs by increasing the amplitude and enhancing the activation of I_{Ks} and partially restoring the APD.¹⁴¹ Kuusela et al¹⁴² demonstrated the hiPSC-CM sensitivity to low extracellular K^+ concentrations and how this could evoke EADs in LQT1 cells. Increased amounts of abnormal Ca^{2+} transients

and mechanical beating behavior have also been reported in addition to slower repolarization.¹⁴³

LQT2 hiPSC-CM studies have recapitulated the clinical finding of AP prolongation in patient-specific hiPSC-CM.^{17,19,20} The in vitro phenotypes in LQT2 hiPSC-CMs in different reports have included a decrease in I_{Kr} , APD prolongation, EADs, and increased drug sensitivity.^{17,19,20,144–147} The in silico investigation of symptomatic and asymptomatic LQT2 individuals suggested that the EAD generation and repolarization failure mechanism in the LQT2 hiPSC-CMs was the reactivation of I_{CaL} .¹⁴⁶ Nifedipine, an L-type Ca^{2+} channel blocker, has been studied with LQT2 hiPSC-CMs, and it has caused a significant shortening of both APD and corrected FPD.^{19,145} Mehta et al¹⁴⁴ showed that lumacaftor, a clinically used drug acting as a chaperone during protein folding, was able to reverse the LQT2 phenotype.

In LQT3, I_{Na} inactivation is compromised, or channel reopening occurs, leading to I_{NaL} , which persists throughout the entire AP and ultimately causes AP prolongation and proarrhythmia.¹⁴⁸ Terrenoire et al¹⁴⁷ showed that the increased I_{NaL} was responsible for the proarrhythmic phenotype and recapitulated the clinical phenotype of the patient with prolonged AP in LQT3 hiPSC-CMs. Overall, hiPSC-CM models of LQT3 have revealed alteration of I_{Na} biophysical properties leading to increased late I_{Na} . In addition, APD/FPD prolongation and EADs have been detected.^{40,56,147,149} Ma et al⁵⁶ showed that mexiletine reduced I_{NaL} and APD in LQT3 hiPSC-CMs. The beneficial effect of mexiletine was also evident in the Malan et al's¹⁵⁰ study, where it was found to shorten APD and FPD and antagonize EADs in a dose-dependent manner in LQT3 hiPSC-CMs.

Recently, computational studies successfully tried to replicate in hiPSC-CMs specific mutations causing the most common LQTS types: R190Q in *KCNQ1* (LQT1)^{41,151} and N996I in *KCNH2* (LQT2)⁴¹ or V1763M in *SCN5A* (LQT3).⁶⁹ Their common denominator is that the effect of the aforementioned channelopathies was studied by means of in silico populations, highlighting the electrophysiological substrates (in terms of ion current maximum conductances) that could make hiPSC-CM more or less sensitive to LQT syndromes and responsive to pharmacological therapies.

Some less common LQT syndromes that have been studied with hiPSC-CMs include LQTS7-Andersen-Tawil syndrome¹⁵² and LQTS8-Timothy syndrome.¹⁵³ hiPSC-CMs from LQT8 patients have been shown to mimic the electrical phenotype of the patient with prolonged APD. CMs also showed DADs, altered Ca^{2+} , and roscovitine which affects I_{CaL} inactivation, reduced APD, restored the irregular Ca^{2+} transient, and decreased the frequency of abnormal depolarizations in these cells.¹⁵³

Brugada Syndrome (BrS)

BrS is an arrhythmic disorder with the manifestation of ST elevation on the surface electrocardiogram (ECG), history of ventricular tachycardia or fibrillation, family history of sudden cardiac death, and syncope.¹⁵⁴ Most of the BrS-related mutations have been found in the *SCN5A* gene, which is encoding the cardiac Na^+ channel and responsible for I_{Na} .¹⁵⁵ The cellular basis for the BrS is believed to be due

to an outward shift in the balance of ionic currents that are active during phase 1 of the AP, in which I_{to} is prominent, exaggerating shortening of APD. Another mechanism behind BrS has been believed to be decreased conduction because of reduced I_{Na} .¹⁵⁶ hiPSC-CMs generated from BrS patients have revealed controversial results. On the one hand, these cells have shown decreased I_{Na} density, ie, the assumed BrS phenotype,^{149,157} triggered AP activity, and abnormal Ca^{2+} transients, which have been normalized after the mutation correction of the patient-derived cells.¹⁵⁷ Ma et al⁵⁶ concluded that a repolarization deficit could be a mechanism underlying BrS after they demonstrated that a severe I_{Na} deficiency could lead to remodeled baseline APs vulnerable to heart rate-induced, I_{to} -sensitive proarrhythmic increased phase 1 repolarization changes. On the other hand, others have not found electrophysiological abnormalities consistent with BrS-like decrease in I_{Na} ¹⁵⁸ or changes in the other currents proposed to contribute to BrS (I_{to} or I_{CaL}).¹⁵⁸ These authors have concluded that the phenotype of these patients is not caused by defects in ion channels or their modulatory subunits but may instead involve the development of structural changes such as fibrosis.¹⁵⁸

Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

CPVT is a hereditary arrhythmic disorder and triggered by emotional stress or physical exercise, causing premature ventricular contractions, and polymorphic VT in a structurally normal heart. In CPVT, stress-induced releases of catecholamines cause dysfunction of Ca^{2+} cycling in CMs, which induces ventricular arrhythmias that can cause ventricular fibrillation.^{159,160} CPVT cases are usually caused by mutation in RYR2¹⁶¹ through different mechanisms. These mutations can lead to hyperphosphorylation of RYR2 and unbinding of stabilizing calstabin2, lowered threshold for store overload-induced Ca^{2+} release, increased sensitivity to RYR2 activators, and unzipping of interdomain interactions.¹⁶² Also mutations in CASQ2,¹⁶³ the major Ca^{2+} storage protein in the SR, and triadin (TRDN),¹⁶⁴ a transmembrane SR protein and component of Ca^{2+} homeostasis, are causing CPVT. It has been suggested that CASQ2 mutation may lead to a reduced direct inhibitory effect on RyR2 leading to spontaneous Ca^{2+} release, to a loss of Ca^{2+} buffering or to remodeling of SR structure and proteins. TRDN mutations are believed to cause CPVT by an impaired calstabin2–RyR2 interaction or a reduction of CASQ2, and also absence of TRDN has been linked to CPVT symptoms.^{164,165} Altogether, it is believed that these CPVT mutations spontaneously increase or cause a leak of SR Ca^{2+} through RYR2, transiently elevating the intracellular Ca^{2+} that can activate I_{NCX} .¹⁶² This generates a depolarizing current that can trigger EADs or DADs, which are believed to underlie the polymorphic or bidirectional ventricular tachycardia.¹⁶² Several groups have reported the generation of CPVT-specific hiPSC-CMs^{24,25,45–47,166–171} mostly with RYR2 mutations, recapitulating the pathophysiological characteristics of the disease. CPVT hiPSC-CMs have been studied with the administration of the beta-adrenergic agonist with mainly voltage clamp and Ca^{2+} imaging, showing abnormal Ca^{2+} handling, spontaneous

Ca^{2+} sparks, and depolarization abnormalities that result in DADs,^{24,25,45–47,167–172} EADs, and spontaneous bursts/tachyarrhythmia.^{24,168} In addition, reduced SR Ca^{2+} content^{24,47,171} and prolonged spontaneous Ca^{2+} sparks have been reported in CPVT hiPSC-CMs when compared with controls.^{46,170} These previous studies implicate that enhanced diastolic Ca^{2+} release/leak and possibly impaired SR Ca^{2+} loading could be the mechanisms for the arrhythmias in CPVT hiPSC-CMs, as contrary to SR Ca^{2+} overload, which has been proposed as one mechanism in studies of isolated CMs from transgenic mice.^{173,174}

Also CASQ2 mutations have been studied with hiPSC-CMs, and Novak et al showed that β -adrenergic stimulation caused DADs, oscillatory arrhythmic prepotentials, after contractions, and diastolic cytosolic Ca^{2+} concentration rise.¹⁷⁵ In addition, the efficacy of the virally mediated gene therapy has been studied in hiPSC-CMs generated from a CPVT-CASQ2 patient and a decrease in the percentage of DADs, re-establishment of the Ca^{2+} transient amplitude, and normalization of the density and duration of Ca^{2+} sparks were observed.¹⁷⁶ Studies about TRDN mutation-related CPVT with hiPSC-CMs have not been reported.

CPVT hiPSC-CM findings have also been validated in the index patients. Kujala et al²⁴ reported that hiPSC-CMs derived from a CPVT patient exhibit EADs and confirmed the presence of EADs in monophasic AP recordings in the same CPVT patient. Paavola et al¹⁶⁶ compared the phenotype between CPVT hiPSC-CMs and the donor patient and demonstrated increased nonalternating variability of Ca^{2+} transients in the CMs and increased short-term variability of the QT interval in ECGs. In addition, it was shown that β -agonists slowed depolarization in RyR2-mutant CMs and monophasic AP recordings of CPVT patients.¹⁶⁶

CPVT hiPSC-CM models have also been used to test new therapeutic options. Di Pasquale et al⁴⁶ showed that treatment of CPVT hiPSC-CMs with KN-93, an antiarrhythmic drug that inhibits Ca^{2+} /calmodulin-dependent serine-threonine protein kinase II (CaMKII) reduced the presence of DADs, suggesting that CaMKII phosphorylation of downstream targets could be central to disease pathogenesis. The arrhythmogenic phenotype of CPVT hiPSC-CMs, including Ca^{2+} handling abnormalities, EADs, and triggered beats, have been rescued with dantrolene, an inhibitor of Ca^{2+} release through RyR2 channels and effect of the drug confirmed with a clinical study.^{47,167} RyR2 mutation-specific dantrolene responses were demonstrated with 6 different mutations when it suppressed the Ca^{2+} cycling abnormalities only in CPVT hiPSC-CMs where RyR2 mutation was in the N-terminal or central region of the protein, and this was consistent with the same patient in silico outcome.¹⁶⁷ This could indicate that the arrhythmogenic mechanisms may vary between mutation locations. The effect of flecainide^{25,169,170,172} and β -blockers^{25,168,169,172} have been studied with several CPVT hiPSC-CM models. Itzhaki et al²⁵ showed that flecainide and thapsigargin (inhibitor of the SR Ca^{2+} -ATPase) eliminated afterdepolarizations, and β -blockers reduced adrenergic stimulation-induced Ca^{2+} abnormalities. Preiginger et al¹⁷⁰ showed that the β -blocker treatment with nadolol during β -adrenergic stimulation achieved a negligible

reduction of Ca^{2+} wave frequency and failed to rescue Ca^{2+} spark defects in CPVT hiPSC-CMs. By contrast, flecainide reduced both frequency and amplitude of Ca^{2+} waves and restored the frequency, width, and duration of Ca^{2+} sparks to baseline levels. Pharmacological studies by Maizels et al¹⁷² revealed the prevention of arrhythmias by propranolol and carvedilol, flecainide, and the neuronal sodium channel blocker riluzole, and suppression of abnormal Ca^{2+} cycling by the ryanodine stabilizer JTV-519 and carvedilol. Pölonen et al¹⁶⁹ studied the antiarrhythmic effect of carvedilol and flecainide with 3 RyR2 mutations. Both drugs lowered the intracellular Ca^{2+} level and the beating rate of the CMs. However, their arrhythmia-abolishing effect was between 30% and 52% and therefore not considered as an optimal effect on the Ca^{2+} level. Carvedilol showed stronger antiarrhythmic effect than flecainide on one of these RyR2 mutations in patch-clamp studies. To conclude, the drug effects on CPVT hiPSC-CMs indicate mutation-specific differences in disease and arrhythmogenic mechanisms of CPVT.

APPLICATIONS OF hiPSC-CMs

Assessing Drug Cardiac Safety and Toxicity

The potential of hiPSC-CMs to assess the occurrence of drug-induced arrhythmias, and consequently as safety pharmacology tools, has been clear since their discovery, and it was intercepted within the CiPA initiative as one of its pillars (<https://cipaproject.org>). In brief, CiPA foundations are (1) assessing drug effects on multiple human cardiac currents, (2) evaluating the arrhythmic risk on the human ventricular cellular electrophysiology first through in silico models, second (3) in vitro by means of hiPSC-CMs, and finally (4) in vivo with ECG biomarkers during phase 1 clinical trials.⁶ Therefore, it is not surprising that studies aimed to demonstrate the ability of hiPSC-CMs as model systems to detect electrophysiological effects of drugs, including delayed or altered repolarization, have increased markedly in recent years. There are also ongoing efforts to define the general principles for the development, validation, and employment of in silico models to be used for TdP-risk, which apply more generally to any proarrhythmia risk assessment.⁷⁶

Several studies have focused on evaluating TdP risks with hiPSC-CMs. Blinova et al²⁶ tested hiPSC-CMs for improving TdP arrhythmia risk prediction of drugs within CiPA. They also showed that concentration-dependent analysis comparing hiPSC-CMs with clinical trial results demonstrated a good correlation between drug-induced rate-corrected APD and FPD increase and clinical trial rate-corrected QT interval prolongation. They tested 14 drugs known for their TdP-risk: arrhythmias occurred with 10 drugs and lack of arrhythmic beating in hiPSC-CMs for the 4 remaining drugs was believed to be due to differences in relative levels of expression of individual ion channels.⁷⁴ Yamazaki et al¹⁷⁷ evaluated the predictivity of drug-induced proarrhythmic risk from MEA data of 28 drugs with varying TdP-risk potentials and provided relative TdP-risk score to each drug by identifying 9 high-risk, 9 intermediate-risk, and 10 low-risk drugs. Navarrete et al¹⁷⁸ demonstrated that

the low-impedance MEA/hiPSC-CM platform could be used to identify and quantify arrhythmic events such as EADs and ectopic beats, the actual underlying mechanisms of TdP, confirming the presence of the phase 2–3 window necessary for inward currents to produce these arrhythmic events. Yamamoto et al¹⁷⁹ showed in the presence of E4031 and cisapride that hiPSC-CMs showed reverse use-dependent FPD prolongation, and categorical analysis was applicable for evaluating torsadogenic risks with FPD and/or corrected FPD. Stillitano et al¹⁸⁰ studied the responses of healthy individuals to a I_{Kr} blocking drug, sotalol, and generated hiPSC-CMs from high-sensitive and low-sensitive subjects. The authors found that sotalol-induced prolongations of FPD and the incidence of arrhythmia were significantly higher in hiPSC-CMs derived from high-sensitive subjects than those derived from low-sensitive subjects.

Given the high interest in hiPSC-CMs as tools to assess drug toxicity, several high-throughput systems, using voltage-sensitive and Ca^{2+} -sensitive probes, optogenetics, and high speed/high resolution microscope, have been developed during the last 5 years. Despite an in-depth review of such systems is out of the scope of this article, we consider worth to mention few illustrative cases. In 2016, Pfeiffer et al¹⁸¹ used kinetic image cytometry and analysis of Ca^{2+} transients on hiPSC-CMs loaded with a fluorescent Ca^{2+} reporter on a training panel of 90 compounds and a follow-up blinded study of 35 compounds (96-well format). They demonstrated that Ca^{2+} transient prolonging drug concentrations correlated with clinically QT-prolonging concentrations better than data from animal tests. In 2016, Klimas et al¹⁸² developed the all-optical electrophysiology OptoDyCE high-throughput system (96-well¹⁸² and 384-well¹⁸³ plate formats), tested on 12 compounds.¹⁸⁴ It combines optogenetic actuation (ie, contactless optical pacing of hiPSC-CMs) and the simultaneous optical sensing of voltage and intracellular Ca^{2+} or dye-free contraction video tracking. In 2017, McKeithan et al¹⁸⁵ developed a high-throughput system (384-well plate format) able to automatically quantify AP biomarkers and drug-induced arrhythmias, exploiting a small molecule voltage sensitive probe and tested on 15 compounds.

Assessing Sex-dependent Differences in Cardiac Electrophysiology

From a clinical perspective, sex-based differences in cardiac physiology are widely recognized, manifesting as poorly diagnosed and too often untreated diseases in the female population.¹⁸⁶ For example, the prevalence of heart failure with preserved ejection fraction is up to 2 times higher in women, whereas heart failure with reduced ejection fraction is more common in men.¹⁸⁶ Similarly, the differences in ECG are also broadly acknowledged; women have longer rate-corrected QT intervals as well as shorter PR and QRS intervals.^{186,187} The underlying mechanisms of differing electrophysiology are, however, poorly understood. This is at least partly because of the scarcity of in vitro and ex vivo data, as access to native human cardiac tissue is limited by ethical aspects. Furthermore, access to female-specific tissue is restricted mainly to postmenopausal women, which seriously limits the interpretation of sex-based differences. The

hiPSC-approach can resolve the problems related to access to human tissue by offering an unlimited source of human CMs. Furthermore, hiPSC-CMs provide a unique way of investigating the contribution of genomic versus hormonal regulation on female versus male phenotype.

Barajas-Martínez et al¹⁸⁸ were the first to use hiPSC-CMs to investigate the sex dependence of arrhythmogenic mechanisms. In their seminal study in 2013, the authors showed that testosterone (1 μ M, 4 weeks) increased the density of I_{to} , by upregulating expression of $K_{v4.3}$ mRNA, providing a potential explanation for the male–female difference in the manifestation of life-threatening arrhythmias that are associated with the J-wave syndromes. More recently, Papp et al¹⁸⁹ showed that in hiPSC-CMs of female origin, estrogen (17- β -estradiol, E2; 1 nM; 1–2 days) increased I_{CaL} by 31% and I_{NCX} 7.5-fold, whereas there was no effect in male CMs. Huo and coworkers¹⁹⁰ reported that E2 (10 nM, 0.5–4 hours) increased the FPD (MEA measurements) and DHT (5 α -dihydrotestosterone, 40 nM, 18 hours) shortened FPD in hiPSC-CMs of female and male origin, respectively. Corresponding findings were reported by Salem et al,¹⁹¹ who showed that acute dihydrotestosterone exposure shortened APD by 57% in male CMs. Furthermore, Huo et al¹⁹⁰ showed that hERG channel block (dofetilide, 0.01 μ M) induced more marked repolarization prolongation and arrhythmias in female-derived than male-derived CMs. The authors identified EADs as the arrhythmogenic mechanism. In line with the weaker repolarization reserve in female hiPSC-CMs, Huo et al showed that male-derived hiPSC-CMs had higher expression levels of KCNE1 gene (encoder of the ancillary β subunit of I_{Ks}) and more pronounced responses to I_{Ks} blocker, JNJ 303. Thus, they demonstrated that hiPSC-CMs can provide mechanistic insights into the increased TdP susceptibility of female patients. The study by Zeng et al¹⁹² corroborated findings of Huo et al¹⁹⁰ showing that hiPSC-CMs from female donors were uniformly more sensitive to hERG channel blockers (dofetilide, 10 nM or cisapride, 300 nM) that prolonged FPD and caused EADs. Whereas, a specific I_{Ks} blocker (L-000768673, 100 nM) induced a more pronounced FPD prolongation in male CMs.

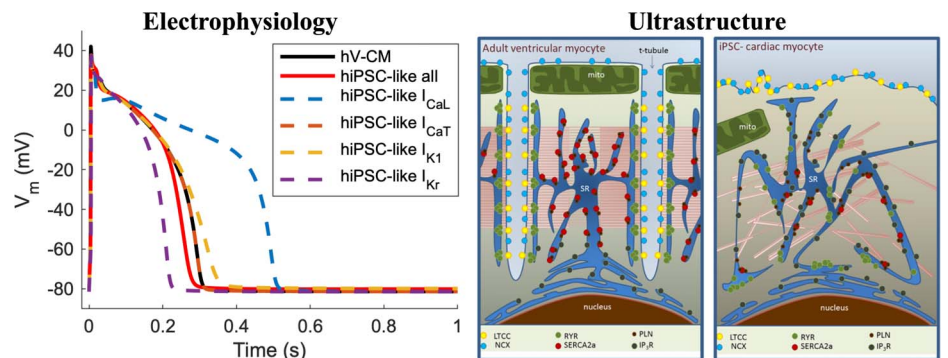
LIMITATIONS OF hiPSC-CMs

Despite the undeniable value of hiPSC-CMs as a human in vitro model, it is fair to recognize that they suffer specific limitations as models fully representative of hV-CMs, as we reported in the previous sections of this review. In this section, we recapitulate and expand on those limitations (Fig. 3).

Some of the ion currents are differently expressed in hiPSC-CMs and hV-CMs. hiPSC-CMs express 2 ion channels that are absent in healthy hV-CMs: I_f ¹⁹³ and I_{CaT} .¹⁹⁴ Furthermore, the balance of repolarizing potassium currents is quite different. The expression of I_{K1} has been reported as low as in human adult atrial CMs (hA-CMs)⁶⁰ or even absent.²⁷ Whereas, the density of I_{Kr} is substantially higher in hiPSC-CMs compared with hV-CMs.^{19,55,193} The balance of I_{K1} and I_f underlies hiPSC-CM automaticity in the voltage clock paradigm; one of the hallmarks of the immature or nonadult hiPSC-CMs. In addition, expression of some ion channels and consequent ion current densities seems to be highly variable in comparison with hV-CMs: lower⁷⁴ or similar¹⁹³ $SCN5A$ and lower⁷⁴ or similar¹⁹³ $KCNQ1$. A huge range of I_{CaL} densities has been reported for hiPSC-CM: 3.3–17.1 pA/pF,^{39,153} which is likely because of different methodology. The only study comparing I_{CaL} under identical experimental conditions showed \sim 50% larger densities in hiPSC-CMs versus hV-CMs.¹⁹⁴ Together, the aberrant balance of inward and outward ion currents reduces the repolarization reserve in hiPSC-CMs compared with their native counterparts. For example, Blinova et al⁷⁴ correlate these differences in expressions to the different proarrhythmic effects that a set of drugs (namely, bepridil, ranolazine, and mexiletine) has in hiPSC-CMs. Furthermore, Lemoine et al¹⁹³ showed that EHT is much more prone to EADs in comparison with human ventricular tissue.

Ca^{2+} handling is functional but not fully developed in CMs derived from pluripotent stem cells.¹⁹⁵ Two main points are worth to be mentioned. First, the lack or lower expressions of functional proteins, eg, junctin and triadin (to facilitate RyR function), and of calsequestrin for Ca^{2+} buffering,

FIGURE 3. Distinct electrophysiological and ultrastructural characteristics of hiPSC-CMs in comparison with human native/adult ventricular CMs (hV-CMs). Impact of prototypical hiPSC-like I_{CaL} , I_{K1} , and I_{Kr} densities, which are 1.5-fold, 0.5-fold, and 4-fold in comparison with hV-CM, on the repolarization reserve, simulated as in Ref. 193. The absence of t-tubules in hiPSC-CM results in spatial uncoupling of L-type Ca^{2+} channel (LTCC) and ryanodine receptors (RyR), as well as irregular distribution of SR Ca^{2+} handling proteins: RyR, Ca^{2+} ATPase (SERCA), and its regulatory protein phospholamban (PLN). Furthermore, inositol triphosphate receptors (IP3R) activity is substantially higher in hiPSC-CMs. The panels for were adapted from Ref. 196 CC BY 4.0 (original Fig. 1, <http://creativecommons.org/licenses/by/4.0/>).



as well as lower SERCA and RyR. Second, hiPSC-CMs lack a well-developed T-tubule network that is a hallmark of mature ventricular CMs.¹⁹⁶ This ultrastructural immaturity results in an U-shaped Ca²⁺ wavefront with a remarkable delay in peak between the cell periphery and center,¹⁹⁷ as observed also in hA-CMs, and a poor excitation–contraction coupling.¹⁹⁸

The hiPSC-CM contraction is also nonoptimal, not only because of poor excitation–contraction coupling, but also to the immature sarcomeric structure and the orientation in multiple directions of the myofibers within the cell.⁹ When compared with native human ventricular tissue, hiPSC-based EHTs lack a positive force–frequency relation,⁶⁸ which is one of the hallmarks of cardiac contractility. Furthermore, the frequency-dependent acceleration of relaxation is much weaker in EHTs.⁶⁸

Finally, the hiPSC-CM potential to recapitulate the contractile and remodeling signaling of adult CMs is not fully known. Jung et al¹⁹⁹ studied the different stages of hiPSC-CMs and showed that at day 30 β 2-AR signaling is dominant, reflecting a relative immaturity of hiPSC-CMs. By day 60, a transition to downstream signaling by the β 1-AR represents a more “adult-like” phenotype. Even by day 90, β 1-AR expression does not fully recapitulate the pattern seen in adult human ventricles, and β -ARs only activate CaMKII at this time, although the protein expression reached a maximal level by day 30. The differential maturation of intracellular signaling pathways in hiPSC-CMs should be considered in disease modeling and drug testing.

CONCLUSIONS, FUTURE DIRECTIONS

In this review, we showed how hiPSC-CMs can recapitulate arrhythmia mechanisms observed in adult CMs, such as abnormal impulse formation and conduction abnormalities, as well as disease phenotypes because of channelopathies (eg, LQT syndrome) or cardiomyopathies (eg, HCM and DCM) that trigger arrhythmic behaviors. However, it can be argued that thus far they have provided only limited new insights into arrhythmia mechanism. This is because, in addition to the specific phenotype-related discrepancies summarized in section Applications of hiPSC-CMs, there are open questions, including the following:

1. How can we improve and standardize the culturing methods so that quantitative interlaboratory or even interbatch comparisons of experimental findings would be possible? Can we establish good manufacturing practices and quality control assays for in vitro experiments toward that goal?
2. How can we overcome the fluidity and variability in the hiPSC-CMs, because of the constantly developing phenotype and lack of chamber/subtype specificity of the CMs? Can the in vivo maturation factors be better mimicked by means of chemical, electrical, and mechanical modulators?
3. There is also a very practical impediment related to the immature phenotype of hiPSC-CM: spontaneous activity is commonly used as a marker of cell viability. Does this not create a baseline bias toward less maturity and robustness?
4. In terms of disease modeling, the controls are also an issue how is the comparison performed and what is a good

control? One challenge in arrhythmia mechanism studies related to disease modeling is that the healthy controls may contain mutations and polymorphisms that cause phenotypic differences between diseased cells and controls. Isogenic controls, however, could more accurately depict nondiseased cellular phenotypes when compared with healthy controls, but generation of these lines is laborious, which are also subjected to clonal variation.

5. Can we expand the hiPSC-CMs’ domain of application from cardiac drug safety to efficacy in a systematic way? The CiPA initiative proposed a consistent framework to exploit in vitro hiPSC-CMs and in silico models, to predict cardiotoxicity of new compounds. Given the patient and disease specificity of conveniently produced hiPSC-CMs, they hold the extra potential to optimize therapies against specific conditions.

As with to other mammalian species and CM subtypes, biophysics-based mathematical models have been developed to represent the specific physiological features of hiPSC-CMs. Such mechanistic models provide the most systematic means for (1) interpreting the highly variable in vitro results from different laboratories⁶⁹ and (2) translating the hiPSC-based findings to the real-human context.¹¹⁰ Especially, the electrophysiological properties of hiPSC-CMs are captured well with the current in silico approaches, so they are potentially valuable for investigating arrhythmia mechanisms. As discussed in section Spatial Dispersion of Repolarization and Myocardial Heterogeneity, computational modeling can provide the means to extrapolate the cell level in vitro findings to investigate tissue level phenomena. More importantly, the in silico approach has already been successfully used as a tool to investigate translatability of in vitro drug and disease modeling data to the human context.^{50,108–110} Also, the characteristic huge variability of hiPSC-CM physiology observed in experiments can be captured and explored computationally, using the populations of models method.^{41,49,50,69,70} There are ongoing efforts to include the description of contractile function in hiPSC-CM models. With these advancements, it will be possible to (1) analyze in silico the impact of the peculiar cell shapes and disorganized sarcomeric structure of hiPSC-CMs on the mechanical phenotype and (2) translate the in vitro contractility findings from of hiPSC-CMs to the human context. Finally, it is widely acknowledged that hiPSC-CMs are constantly developing and/or maturing. This phenotype fluidity can be replicated in silico either as (1) snapshots like Paci et al²⁰⁰ already did for their hESC-derived CM model or as (2) dynamic processes accounting for the changes in structures and protein expressions over time. This kind of quantitative description of the phenotypic continuum would make the translation of hiPSC-CM findings much more reliable and transparent.

Simultaneous quantification of APs (transmembrane voltage), calcium transients (intracellular concentration fluctuations), and contractility (contraction and/or force of contraction) is one of most significant recent technological advances. Ability to mechanistically investigate the interdependencies between these principal outputs of CMs provides unprecedented insights into the phenomena that underlie

arrhythmias. Another giant methodological leap is related to the so-called organ-on-chip or body-on-chip approaches. Within a few years, these platforms have the potential of recapitulating, for example, (1) a more mature CM phenotype, because of more physiological environment, (2) the complex tissue structures, including not just CMs as the only cell type, and (3) neural modulation of arrhythmia substrates, triggers, and automaticity. That would substantially strengthen the translational link between in vitro findings of the arrhythmogenic mechanisms versus arrhythmia formation and the management in the whole organ/organism.

If the methodological capabilities and advances described above, as well as the to-be-realized future advances in in vitro technologies and approaches, can build on improved consistency, reproducibility, and phenotypic maturity, there is a great opportunity to harness the full potential of hiPSC-CMs for investigating arrhythmic mechanisms, including the perspectives of cardiotoxicity and efficacy testing of drug compounds, as well, the development of patient-specific models, and personalized treatment.

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