Assessment of Cardiotoxicity With Stem Cell-based Strategies

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ABSTRACT

Purpose: Adverse cardiovascular drug effects pose a substantial medical risk and represent a common cause of drug withdrawal from the market. Thus, current *in vitro* assays and *in vivo* animal models still have shortcomings in assessing cardiotoxicity. A human model for more accurate preclinical cardiotoxicity assessment is highly desirable. Current differentiation protocols allow for the generation of human pluripotent stem cell–derived cardiomyocytes in basically unlimited numbers and offer the opportunity to study drug effects on human cardiomyocytes. The purpose of this review is to provide a brief overview of the current approaches to translate studies with pluripotent stem cell–derived cardiomyocytes from basic science to preclinical risk assessment.

Methods: A review of the literature was performed to gather data on the pathophysiology of cardiotoxicity, the current cardiotoxicity screening assays, stem cell–derived cardiomyocytes, and their application in cardiotoxicity screening.

Findings: There is increasing evidence that stem cell–derived cardiomyocytes predict arrhythmogenicity with high accuracy. Cardiomyocyte immaturity represents the major limitation so far. However, strategies are being developed to overcome this hurdle, such as tissue engineering. In addition, stem cell–based strategies offer the possibility to assess structural drug toxicity (eg, by anticancer drugs) on complex models that more closely mirror the structure of the heart and contain endothelial cells and fibroblasts.

Implications: Pluripotent stem cell–derived cardiomyocytes have the potential to substantially change how preclinical cardiotoxicity screening is performed. To which extent they will replace or complement current approaches is being evaluated. (*Clin Ther*. 2020;42:1892−1910) © 2020 Elsevier Inc.

Key words: cardiotoxicity, drug testing, stem cells, toxicology.

INTRODUCTION

Drug-related cardiotoxicity is a problem for both the affected individual and the development of novel drugs. Cardiotoxicity tests to date are insufficient because they occasionally fail to identify toxic drugs, leading to potential harm to humans in clinical studies and during later use. Moreover, current cardiotoxicity assays also regularly prematurely classify novel drugs that might be safe in humans as toxic. These problems can partially be attributed to insufficient animal models, making cardiotoxicity screening with human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) an attractive new alternative. This review article explores recent developments, biological findings, technological and regulatory aspects, and the chances and shortcomings of stem cell–based strategies for cardiac drug testing.

Drug testing has been around for tens of thousands of years. The Neanderthals have been reported to have chewed poplar bark, which contains the anti-inflammatory compound salicylic acid.[1] These unguided practices have continued until far after the Middle Ages, resulting in the word *quack*, a fake

physician, originating from the Dutch word *kwakzalver*. However, this word only got a negative meaning during the 16th century, after the knowledge spread that most drugs used by quacks did not have a proven effect. Only in the 20th century did drug testing become more extended and regulated, when the Federal Food Drug and Cosmetic Act passed in the US in 1938, obligating drugs to undergo animal testing before they can be put on the market. This act was preceded by the deaths of >100 people after the poisonous diethylene glycol was used as a solvent for sulfanilamide and the product was marketed by an American pharmaceutical company.[2] Thus, although pharmacological testing on animals can be traced back to the 2nd century to the Greek physician Galen, only the 1938 act made it obligatory in the United States.[3] The postmarket monitoring of drugs came into effect even later, only after gynecologist and obstetrician William G. McBride reported severe teratogenic effects of the drug thalidomide in 1961.[4,5] These findings also made it mandatory to test drugs on pregnant animals before marketing. The drug was withdrawn from the UK market in 1961 and from the world market in 1962 after having caused an estimated 10,000 birth defects.[3] Finally, the unification of rules and regulations concerning drug testing among the different member states of the European Union happened in 1995, when the European Medicines Evaluation Agency (EMEA, later EMA) was founded. Currently, (cardio)toxicity screening is mandatory during preclinical drug development and regulated by the EMA and the US Food and Drug Administration (FDA), respectively (International Conference on Harmonization [ICH]; eg, S7B Non-clinical Evaluation of the Potential for Delayed Ventricular Repolarization [QT Interval Prolongation] by Human Pharmaceuticals).[6,7]

Animal models are a core feature of (cardiac) drug testing, and there are many examples in which animal models accurately predicted drug toxicity (eg, nephrotoxicity of cyclosporine and peripheral neuropathy under isoniazid treatment[8]). These models faithfully recapitulate tissue composition and spatial organization. Many aspects of cardiotoxicity will likely be difficult to model without animal use, such as the toxicity of liver metabolites and toxicity via inflammation, to name only a few examples. However, there are several concerns. First, ethical

concerns eventually led to the integration of the 3R's in 2010 by the EMA. The 3R's stand for *replacing* animal testing with nonanimal methods, *reducing* the number of animals used (while still obtaining significant data), and *refining* the test to reduce stress and improve the welfare of the animals used for testing. Rodents are most commonly used for animal testing, including rats, mice, and guinea pigs. Larger animals (pigs, dogs, sheep, and primates) are physiologically closer to humans. However, the ethical and economical dilemma with using these animals is usually bigger, explaining why the use of these species is generally minimized. The second concern is interspecies differences. Animal models differ from human models in terms of physiology and metabolism. These differences exist in both healthy models and disease models, which can lead to differences in drug effects between animals and humans.[5,9] There are 2 ways in which animal models can fail to predict toxicity for humans, either by falsely qualifying a drug as safe or by wrongly qualifying a putatively effective drug as toxic.[10] A prime example for the first case scenario is the abovementioned drug thalidomide, which resulted in tragic embryopathy in humans but did not have teratogenic effects in mice. Thalidomide (like other immunomodulatory drugs, eg, lenalidomide) may bind to the protein cereblon, thereby activating the cereblon E3 ubiquitin ligase complex. It was only realized in retrospect that we might have missed the tragic consequences in the preclinical mouse model because of a single amino acid difference in cereblon between mice and humans.[11] Hence, this drug was the first to raise awareness that interspecies differences exist with regard to drug effects, and eventually drug testing in multiple animal species was made obligatory.[4] Moreover, it is now mandatory to perform drug testing on at least 1 rodent and 1 nonrodent species as part of routine toxicology tests.[12] Thalidomide was not the only drug that was well tolerated in animals but had deleterious consequences in humans. Theralizumab, a superagonist CD28 monoclonal antibody, which was intended for the treatment of chronic lymphocytic leukemia and rheumatoid arthritis, is of particular interest because the consequences of its first in human application changed the European guidelines to mitigate risks for first-in-human clinical trials. Theralizumab had been applied in nonhuman

primates without incident[13]; however, the infusion in 6 healthy volunteers during a Phase I trial induced a massive cytokine storm. All 6 patients had to receive prolonged intensive care treatment.[14] There are more examples, such as BIA 10-2474, a fatty acid amide hydrolase inhibitor that caused cerebral hemorrhage in 5 patients (1 of whom died) after it had been applied in dogs without incident.[15]

Strikingly, almost 90% of all new drugs fail clinical trials, and half of these failures are attributed to toxicity that had not been detected in preclinical trials.[16,17] Other studies report similar numbers. Data for Phase II and Phase III studies that failed between 2013 and 2015 (214 in total, with 174 of these having a reported cause of failure) indicated that in Phase II trials 25% failed because of safety problems, whereas in Phase III trials 15% failed because of safety concerns.[18] Conversely, it is unknown how many potentially effective substances are not being developed further because they are falsely qualified as toxic. Drug development takes 10−15 years and costs approximately $1000 million,[19] indicating that a huge amount of time, effort, and money is lost for every new drug that has to be abandoned. It has been calculated that terminating 5% of all studies that fail in Phase III trials during Phase I trials could reduce drug developmental costs by approximately 5.5 to 7.1%,[20] and this is not even accounting for the loss of potential new therapeutics. Model systems that better predict human toxic effects are therefore highly desirable.

Cardiotoxicity

A systematic review identified 462 medicinal products that were withdrawn from the market between 1953 and 2013. Hepatotoxicity was the most common reason for market withdrawal (n = 81). Cardiotoxicity was the third most common reason (n = 63).[16] Another study reported similar numbers. In this study, adverse cardiovascular drug effects were the fourth most common reason for drug withdrawal from the market (61 of 464) between 1950 and 2017.[21] Importantly, only 13 (21%) of the drugs withdrawn because of cardiovascular toxicity were on the market as cardiovascular drugs, and some of the withdrawn drugs had been in the market for >40 years, including isoprenaline. These numbers viewed with the fact that cardiac toxicity is potentially lethal highlight the importance of reliable models for cardiotoxicity-related drug testing.[22] Arrhythmogenicity represents the classic (clinically acute) mode of cardiotoxicity. Other toxic effects on cardiomyocyte structure and function only came into focus more recently, mainly as consequences of the development of new classes of anticancer drugs.

Arrhythmogenicity is most often caused by the block of a potassium channel, surfacing as QT prolongation on the ECG, and can lead to life-threatening torsades-des-pointes (TdP) tachycardia (Figure 1). The *HERG* gene encodes for $K_v11.1$, the α-subunit of a potassium channel (often called hERG channel) that is responsible for the rapidly activating component of the delayed rectifier current I_{Kr}, the main repolarizing current. The activation initiates repolarization and thereby terminates the plateau phase of the action potential.[23,24] *HERG* gene mutations cause long QT syndrome 2.[25] A prominent example of a drug that blocks I_{Kr} is the nonopioid antitussive drug clobutinol, which was widely used until 2007, when the EMA recommended it be withdrawn from the market.[22,26,27] In 1999, an 11-year-old boy (who was already diagnosed with long QT syndrome) developed TdP tachycardia while being treated with clobutinol. However, it was not until 2004 that clobutinol's arrhythmogenic potential was found. *HERG* block has led to the market withdrawal of several substances (eg, the antihistaminic drug terfenadine[28] and the gastric prokinetic agent cisapride[29]) during the past few decades. Preclinical safety assessment, therefore, focuses on the early detection of arrhythmogenicity. Torsadogenic risk assessment relies on *in vitro* and animal models as well as clinical data (eg, from the FDA's Adverse Event Reporting System) and is combined into 4 risk categories (known risk, possible risk, conditional risk, and drugs to avoid in congenital long QT syndrome[30]). Several preclinical assays are approved by the EMA and the FDA. These assays are complementary, and the combination of test systems is recommended because every test system currently in use has shortcomings. I_{Kr} assays analyze drug action in heterologous hERG channel expression systems (non-cardiomyocytes). These assays are technically rather simple (eg, automated voltage clamp, flux assays, fluorescence, and radioligand binding assays[31]), can be performed in medium to high throughput, and are therefore regularly included early in drug development.
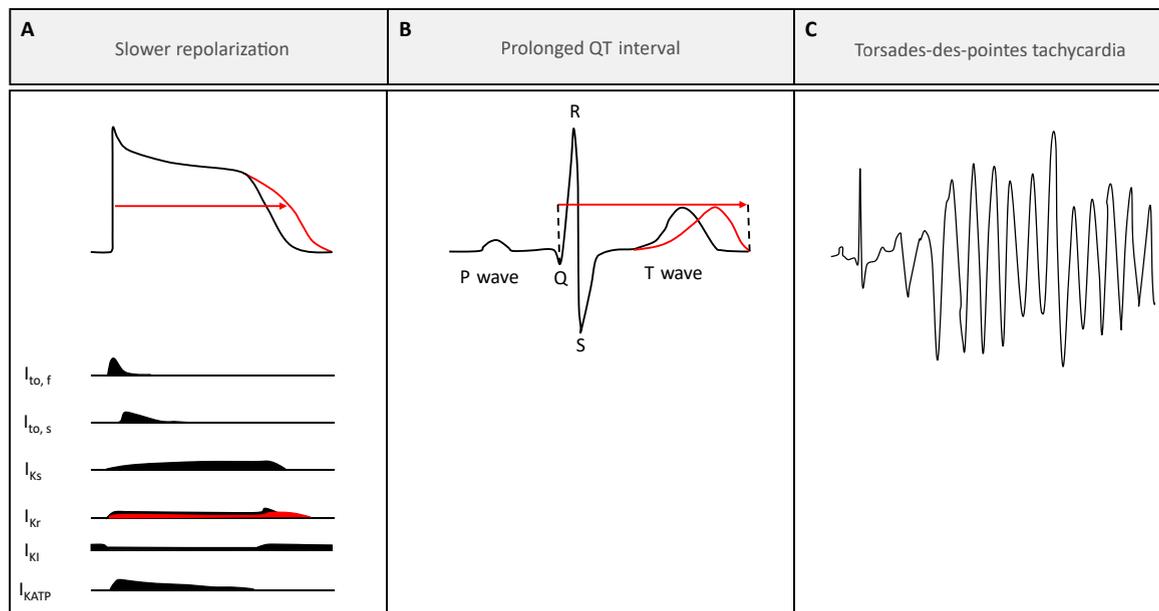
Figure 1. The effect of a hERG block on ventricular action potentials and the related ECG signals. (A) Regular and slowed ventricular action potential attributable to hERG block. (B) Effect of hERG block on the ECG. (C) Onset of torsades-des-pointes tachycardia.[38]

However, risk prediction by I_{Kr} assays is not optimal. On the one hand, drugs that block hERG in this artificial system may have a low (or even no) risk to induce arrhythmia. Nevertheless, these drugs are abandoned early during drug development. Some studies have reported that >75% of all substances fail the hERG assay.[32] On the other hand, some drugs have an inherent arrhythmogenic risk that is not detected by I_{Kr} assays (eg, ciprofloxacine and diphenhydramine).[33] There are several likely explanations for this discrepancy. First, cardiomyocytes are often able to compensate hERG channel blockade by modulating other ion channels, a phenomenon that is described by the term *repolarization reserve*.[31,34] Second, in contrast to classic textbook classifications, many drugs are not simple hERG blockers but have a multi−ion-channel profile (which is often called *dirty* in pharmacology). Verapamil, for example, blocks hERG channels (action potential prolongation) but does not prolong QT interval in humans because it simultaneously blocks the L-type calcium channel (action potential shortening),[33] highlighting the need for test systems that more closely model cardiomyocyte physiology, or even better the entirety of the 3×10^9 cardiomyocytes in a human heart[35] with all their spatial inhomogeneity. The aforementioned clobutinol case is a good example of additional difficulties in arrhythmogenic risk assessment. Clobutinol only mildly affected the normal action potential but exhibited a pronounced effect on the mutated hERG channel.[26] Other *in vitro* assays, such as Langendorff-perfused canine hearts[36] or Purkinje fiber action potential recordings,[37] can overcome these limitations but are technically challenging, rely on animal use, can only be performed in low throughput, and are not able to assay drug metabolites and long-term effects. Long-term toxicity in particular cannot be assessed because these models rely on *ex vivo* preparations of animal heart and therefore represent dying specimens with very limited survival time. These combined long-term effects can

thus only be assessed in *in vivo* animal models, which, for ethical reasons, should be limited as far as possible.

In vitro assays are even more limited with regard to screening for structural toxicity as caused by anthracyclines and nucleoside reverse transcriptase inhibitors or checkpoint inhibitors. The mode of action of these substances (well established or new) is not well understood.[39] Anthracyclines exert a largely dose-independent cardiotoxic effect that results in acute heart failure but also have a cumulative cardiotoxicity, eventually leading to a decline in heart function. The most common form of tyrosine kinase inhibitor toxicity is a decline in systolic left ventricular function.[40] New checkpoint inhibitors regularly induce myocarditis[41] but can also cause Takotsubo cardiomyopathy and conduction abnormalities.[42] These variable clinical consequences indicate that cardiotoxicity involves not only cardiomyocytes but also, for example, inflammatory cells and therefore represents a major challenge for preclinical toxicity screenings.

hiPSC-CMs for Drug Toxicity Screening

Given these limitations and recognizing the fact that drugs regularly have to be withdrawn from the market because of cardiotoxicity, there is a need for more comprehensive models for drug testing. Adult human cardiomyocytes, obtained, for example, through biopsy or from explanted hearts, can only be obtained from patients with cardiac diseases, are extremely difficult to culture,[12] and do not allow for performing high-throughput experiments. Whereas many organs harbor a stem/progenitor cell population, the heart lacks such a stem/progenitor cell population,[43] and the only source for a large number of human cardiomyocytes are pluripotent stem cells (embryonic stem cells or induced pluripotent stem cells). The use of embryonic stem cells is problematic because of ethical concerns and regulatory restrictions. Most research laboratories therefore work with induced pluripotent stem cells. Modern differentiation protocols allow for generating cardiomyocytes in very large numbers.[44] Regular differentiation protocols give rise to mixed cell populations, with most (>55%, up to almost 100%) being ventricular cardiomyocytes,[45,46] but specific differentiation protocols for atrial and nodal cells have been established.[47–49] Notably, the classification was mainly based on electrophysiologic properties.

hiPSC-CMs are commonly classified as atrial-like, ventricular-like, or nodal-like by the action potential shape and expression of either the ventricular or atrial isoforms of the myosin light chain 2 (MLC2V and MLC2A, respectively). For this purpose, action potential shape can be quantified by its relative plateau shape duration. Action potential duration (APD) at a certain percentage of repolarization (eg, APD90 duration[46,50]) is operationalized and used alone or as a ratio (eg, repolarization fraction [APD90-APD50]/APD90[50] or APD20/APD80[45]) to classify atrial cardiomyocytes, capitalizing on their shorter plateau duration. Although this classification recapitulates staple features of chamber specificity, other aspects, such as protein and gene expression, were often neglected and only more recently came into focus using single-cell sequencing and proteomics analysis.[51,52]

According to the FDA guidelines, to bring a new drug to the market, appropriate toxicity tests have to be performed. These tests include but are not limited to "the studies of the absorption, distribution, metabolism, and excretion of the drug in animals." Currently, the FDA has no regulations on drug testing with hiPSC in *in vitro* models. There are, however, strong endeavours to include pluripotent stem cell derivatives in preclinical drug testing.[53] In 2013, the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative was established. Among others, the steering team of CiPA is composed of members of the FDA, EMA, and health organizations of other countries, including Japan. CiPA focuses on the proarrhythmic risk of drugs before admission to the market based on a "mechanistic electrophysiologic understanding of proarrhythmia." (https://cipaproject.org/about-cipa/) This risk is researched by assessing multiple cardiac ion channels, *in silico* reconstruction of the cardiac action potential[54,55], by confirming these findings using hiPSC-CMs[56,57] and eventually using ECG data from Phase I trials to evaluate so far unanticipated electrophysiologic effects.[58] The overall goal of these additional components of preclinical testing is to use nonclinical data to improve clinical decision making regarding drug discovery. This is the first stage of CiPA, and the goal is to reach this stage by June 2020.

In a perfect setting, mature adult cardiomyocytes would be used for screening. However, hiPSC-CMs are structurally and functionally immature and differ

in several aspects from adult cardiomyocytes.[52,59] Mature cardiomyocytes are rod shaped (cell length, approximately 150 μm; cell width, approximately 20 μm)[60] and have well-developed T-tubules and defined sarcomere organization (sarcomere length, approximately 1.8–2.2 μm). A high percentage are multinucleated and do not proliferate. Physiologic characteristics are a stable membrane potential at –90 mV, no spontaneous beating, and a sodium-driven action potential, followed by a plateau phase that in total lasts approximately 300 msec. Contractility is initiated by a calcium-induced calcium release, and there is a positive force–frequency relation. Adult cardiomyocytes contain a high number of mitochondria, and their metabolism is mainly based on fatty acid oxidation.[61] hiPSC-CMs differ in many of these aspects. They are more circular (approximately 50 μm in width),[62] a high percentage are still in the cell cycle,[63] and their metabolism is mainly based on glucose consumption.[64] These structural differences influence physiologic function; for example, the smaller size results in a much smaller membrane capacitance.[65–67] Most important for the risk assessment of arrhythmogenicity, however, are other physiologic differences. The inappropriate spontaneous beating pattern of the hiPSC-CMs is the most obvious one; others are (1) the less hyperpolarized resting membrane potential (–60 mV),[68,69] (2) slower upstroke velocity,[65,70] (3) shorter action potential, and (4) repolarization relying mostly on I_{Kr}, whereas the role of I_{Ks} is debated.[59,71,72]

Whether these differences will limit the predictive value of hiPSC-CMs for preclinical drug testing remains to be seen, but there are reasons to be optimistic. First, disease-specific hiPSC-CMs recapitulated classic features of monogenic cardiac diseases, which are known to induce arrhythmias (eg, long QT syndrome type 1,[73] long QT syndrome type 2,[74] catecholaminergic polymorphic ventricular tachycardia,[75] and forms of hypertrophic cardiomyopathies associated with long QT syndrome[76]). Second, given the fact that immaturity represents a main limitation, extensive approaches have been developed to mature hiPSC-CMs.[77] These approaches include long-term culture, supplementation with thyroid hormone, and tissue engineering (discussed below). Approaches to further

increase maturation for cardiotoxicity screening seem reasonable: early after depolarizations, for example, mainly occur during bradycardia,[78] but the spontaneous beating of immature cardiomyocytes, usually at rates of approximately 60–80 beats/min, does not allow for applying low-frequency pacing protocols. Second, evidence is increasing that stem cell–derived cardiomyocytes predict arrhythmogenicity with high fidelity. Impedance measurements recapitulated the anticipated effects of 49 well-known inotropes with high sensitivity (90%) and specificity (74%) and accuracy (82%).[79] Specific hERG channel blockers caused arrhythmic beating in such an impedance assay, whereas verapamil did not.[80] Similar results were reported in studies that measured field potentials with multielectrode arrays. Sotalol (hERG channel blocker) concentration dependently increased field potential duration (with a maximal prolongation of 36% at the highest concentration tested) and eventually resulted in notched repolarisation waves, indicating early afterdepolarization.[81] Importantly, sensitivity for most (8 of 9) substances tested was higher (effect seen at 3- to 10-fold lower concentrations at a similar degree of specificity) compared with currently used assays (eg, rabbit cardiac wedge preparations).[82] hiPSC-CMs might also be helpful to detect long-term exposure-related substance cardiotoxicity. For example, terfenadine was withdrawn from the market because of the arrhythmogenic risk. However, there is a difference between the therapeutic antihistaminic concentration (low nanomolar range) and the reported IC_{50} for hERG channel blockade (200–10 mM).[33,83] A large study used Langendorff-perfused rabbit hearts to investigate these discrepancies and to more precisely describe the arrhythmogenic potential of terfenadine. The authors describe a complex time and concentration dependency (generally a higher risk after prolonged exposition but also a further increase in APD after washout). Neither APD nor QTc reliably predicted the occurrence of ventricular tachycardia.[83] Such a study is labor intensive, relies on a large animal number, and is not applicable for standard preclinical testing. Long-term drug exposure in stem cell–derived cardiomyocyte cultures, however, is simple and has a time- and concentration-dependent arrhythmogenic risk for terfenadine beginning after 12–48 h.[80]

Overall, evidence is increasing that hiPSC-CMs can be used to assess cardiotoxicity; however, there are limitations. hiPSC lines are available from cell banks (eg, American Type Culture Collection or European Bank for Induced Pluripotent Stem Cell), but (most) pluripotent stem cell culture protocols are expensive and labor intensive.[69] Similarly, even though directed cardiac differentiation became much easier in the last few years as defined differentiation protocols have been developed (and by now differentiation kits are commercially available), these are also expensive and cardiac differentiation capacity varies among cell lines.[84,85] Even though most academic laboratories perform directed differentiation in house, there is the opportunity to skip this step as stem cell–derived cardiomyocytes become commercially available. A few studies have directly compared different cardiomyocytes from different hiPSC lines head to head[57] (Mannhardt et al, unpublished data, 2020), indicating a surprisingly high degree of variability among physiologic characteristics (eg, relaxation time of 100 to approximately 500 msec). Similarly comparing results from different laboratories (different hiPSC lines and differentiation protocols) found a great variability in physiologic parameters (eg, APD ranging from 150 to 600 msec[86]). Even within individual hiPSC lines, substantial batch-to-batch variability occurred.[87] However, canonical drug effects could be observed in most lines (Manhardt et al, unpublished data, 2020), and comparison of the relative change to the baseline has successfully been applied to compensate for variability in baseline characteristics,[57,88] indicating that this limitation can be overcome. These physiologic differences might reflect differences in the origin of the somatic cells that were used for reprogramming and the reprogramming method, but might also be caused by the directed cardiac differentiation methods and are probably further amplified by differences in the maturation state. Several strategies to overcome stem cell–derived cardiomyocyte immaturity exist.[89,90]

One of these strategies is cardiac tissue engineering (Figure 2), which generates cardiac tissue constructs that more closely mirror the morphologic and physiologic situation in the heart. Because this is a major technique in our department, we discuss this approach in more detail. Cardiac tissue engineering was first described in 1997 by Eschenhagen et al.[91]

Since its first description, this method has evolved considerably in different directions.[92] Currently, hiPSC-CMs are routinely used to create cardiac tissue constructs in different shapes and sizes. There are mainly 3 (at least theoretical) advantages over 2-dimensional (2D) culture: (1) hiPSC-CMs mature in 3-dimensional (3D) culture, (2) tissue engineering enables the combination of several different cell populations (endothelial cells, fibroblasts, but also immune cells) in a more physiologic spatial network, and (3) contractility, which reflects the main function of the heart, can be easily measured in tissue constructs. There are, however, shortcomings of tissue engineering strategies. Miniaturized constructs require only 50,000 cells,[93] but standard cardiac tissue constructs so far are generated from 5×10^5 to 1×10^6 cells.[94] The waiting time until first experiments can be performed is also longer, usually between 5 and 14 days.

Isometric, in particular auxotonic, stress matures hiPSC-CMs.[94,102] Cardiomyocytes cultured in a 3D format are anisotropic and longitudinally aligned, resulting in a lower circularity index. They have higher mitochondria content and exhibit a more regular sarcomere pattern with sarcomere length close to the adult ventricular tissue. In addition, they have characteristics of metabolic[64] and functional maturation.[94] Moreover, recent developments, such as bioprinting, can be used to structure the deposition of individual cell types in multiple cell type models. For example, collagen models resembling the physiologic human heart structure can be 3D bioprinted and loaded with multiple cell types. A particular 3D bioprinting approach is called Freeform Reversible Embedding of Suspended Hydrogels (FRESH), which allowed the manufacturing of functional ventricle-shaped structures.[101] Although structures such as functional heart valves can be printed and loaded with cells, steps still have to be taken to be able to print a functional heart.[103]

In general, basically all 3D tissue constructs allow for easily and reproducibly measuring frequency, force, contraction, and relaxation kinetics, which could be used in an integrated manner to estimate arrhythmogenic potential. First results indicate the feasibility of this approach as exemplified by a drug-induced increase in relaxation time. This effect correlated well with published IC_{50} concentrations.[104]
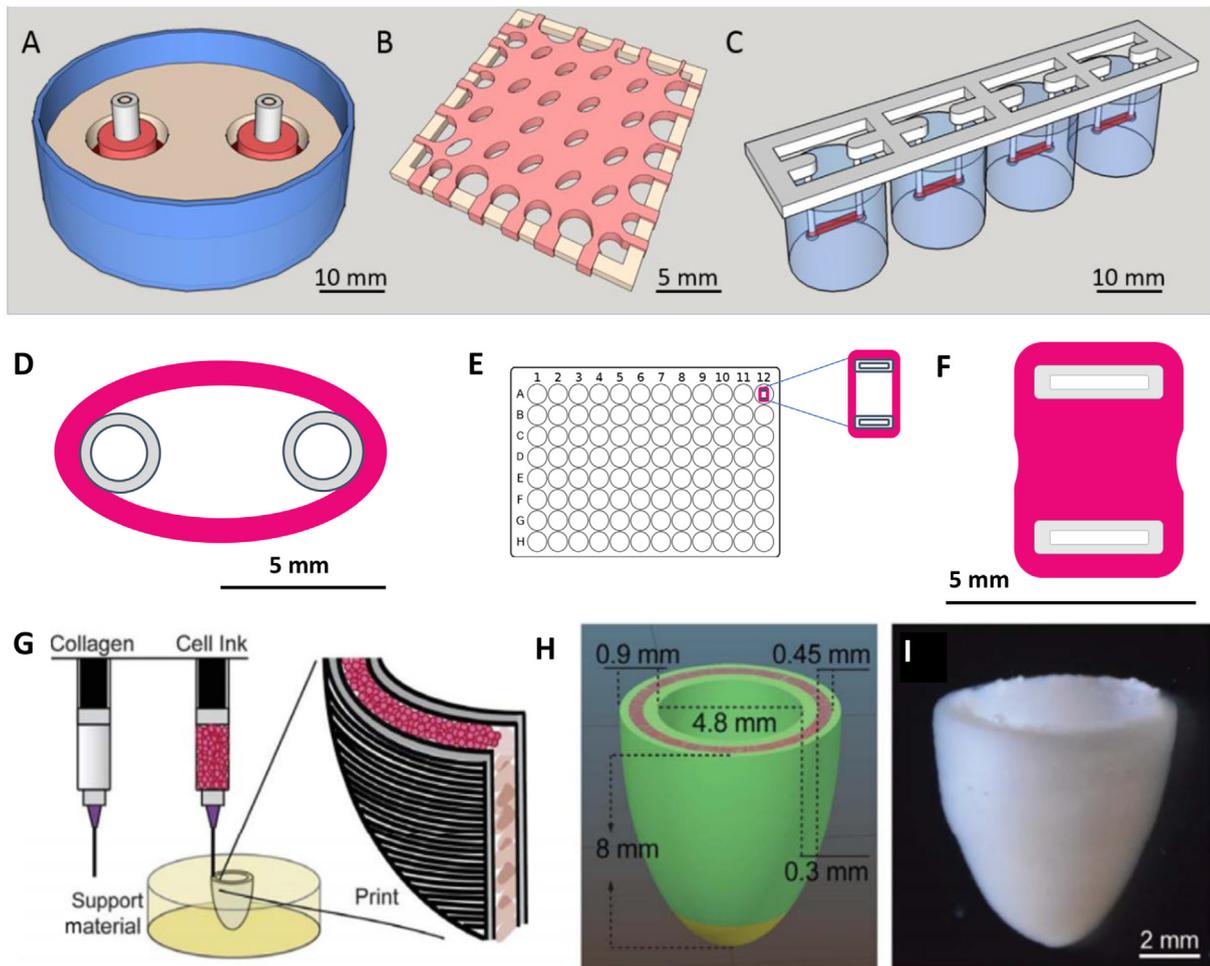
Figure 2. Overview on engineered cardiac tissue models. (A−F) Different formats of engineered heart tissue constructs. (A) Ring-shaped engineered heart tissue.[95] (B) Patch-shaped engineered cardiac construct.[96] (C) Fibrin-based stripe format engineered heart tissue.[97] (D) Ring-shaped engineered human myocardial constructs.[98] (E and F) Miniaturized human cardiac constructs.[99,100] (G) Schematic overview of Freeform Reversible Embedding of Suspended Hydrogels printing with collagen and cell ink. (H) Schematic image of a 3-dimensional (3D) printed ventricle with an internal and external collagen shell (green), cardiac cells (pink), and an only collagen part (yellow). (I) A printed ventricle, as modeled in panel G.[101] Adapted with permission.[92,101]

The engineered heart tissue model found a high prediction accuracy of 93% to predict positive or negative inotropes (after an initial training phase with 8 known drugs) in a blinded, multicenter study compared with 85% for 2D cardiomyocyte monolayers.[105] The use of atrial-like tissue constructs further broadens the spectrum of possible drug screening experiments[46,93,106,107]; for example, the ultrarapidly activating delayed rectifier K^+ current is predominantly expressed in the human atrium. 4-Aminopyridine, an ultrarapidly activating delayed rectifier K^+ current blocker, increased APD in human atrial tissue constructs, whereas ventricular tissues were unaffected.[93] As a limitation, standard assays to measure arrhythmogenic potential are difficult to apply in 3D tissues. For patch clamp analysis,

cardiomyocytes have to be isolated from the tissue, which is labor intensive and requires isolation protocols with limited yield.[108] Sharp microelectrode assays are technically challenging in 3D constructs, but generally feasible.[98,108] However, these factors also do not allow for performing high-throughput assays. Efforts are ongoing to incorporate multielectrode arrays, but the most advanced solutions to date are voltage- and Ca^{2+}-sensing approaches.[106,109-111] Thus, so far, mainly surrogate parameters, in particular contraction kinetics and pattern, are used to overcome the limitations of electrophysiologic accessibility. Besides cardiotoxicity studies, 3D constructs have great potential to screen for new drug candidates.[99,100]

Assessment of Structural Cardiotoxicity With hiPSC-CMs

Clinical manifestation caused by targeted cancer therapeutics and classic anti-cancer drugs, such as anthracyclines, can range from subtle ECG alterations over vascular effects to immediate, marked declines in left ventricular function.[112] The mechanisms by which these drugs exert cardiotoxicity are not well understood, which makes it difficult to assess structural cardiotoxicity. Studies that evaluated structural consequences of tyrosine kinase inhibitor[113] or anthracycline treatment[114] used assays that allow for high-throughput screenings and high-content assays to measure viability and morphology.[115] The metabolic cardiomyocyte immaturity, however, might result in a (partial) resistance to mitochondrial mechanisms of toxicity.[116] Similar to the above mentioned studies on arrhythmogenic risk prediction, the more mature phenotype of cardiomyocytes in the 3D format might offer advantages in this respect, in particular because 3D constructs can be cultured for longer periods. Data from a study with rodent cardiac constructs indicate that contractility parameters could serve as surrogate parameters for structural cardiotoxicity[117] and more recently contractility measurements have been performed with a high-content microscope to assess cardiotoxicity of tyrosine kinase inhibitors.[118] Although cardiomyocytes make up 80% of the mass of the myocardium, they only make up approximately 20% of the cell number, even in the healthy heart.[119] Other cell types present include endothelial cells, resident mesenchymal cells (eg, fibroblasts), leukocytes (myeloid, eg, macrophages, and lymphoid), and a very small number of adipocytes. Classic cardiotoxicity screenings relied on cardiomyocytes only or on whole organisms. Pluripotent stem cells, however, offer the possibility to individually assess the toxicity on different cell types. Whether this is necessary and provides any additional benefit remains to be elucidated. Cardiac tissue constructs so far contain mainly cardiomyocytes (additionally a stromal cell population in some models[120,121]). Recently, though, multi-cell type models have evolved into another area of rapid development. In general, these models are based on cardiomyocytes blended with other cell types. Examples are cardiomyocytes co-cultured with a defined ratio of mixed non-cardiomyocytes[122,123]; cardiomyocytes in co-culture with endothelial cells[124-126]; a co-culture of cardiomyocytes and fibroblasts[98,127-130]; tricellular models consisting of cardiomyocytes, endothelial cells, and fibroblasts[131]; and in vitro cardiac inflammation and injury models consisting of a co-culture of cardiomyocytes with macrophages.[132-135] A model that combines all cell types present in the human myocardium, however, has yet to be developed.

METHODS TO MEASURE ARRHYTHMOGENICITY

A screening assay needs to be technically easy to perform, allow for a high-throughput screening, and to be inexpensive. Cardiac electrophysiology from pluripotent stem cells can be evaluated by multielectrode arrays, patch clamp analysis, impedance measurements,[136] and voltage-sensing optical approaches[137] (Figure 3).

Multielectrode Arrays

Multielectrode arrays record extracellular field potentials of cell populations. Their great advantage lies in their simplicity. They allow for performing high-throughput measurements and have been used for most safety assessment studies so far. By measuring the extracellular field potential, multiple parameters can be determined. These parameters include, but are not limited to, the contraction rate, spike amplitude, and field potential duration. Cardiotoxicity, at least when presenting as arrhythmia-like events, can be deduced from these
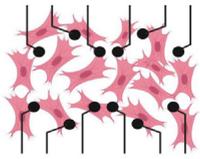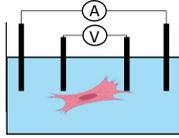
Multielectrode array	Patch clamp	Impedance measurements	Optical techniques

A

B

Multielectrode array

Advantages
- Simplicity
- High throughput
- Generally feasible in 3D constructs

Disadvantages
- Very dependent on outer parameters, such as cell density and maturity

Patch clamp

Advantages
- Cost-effective
- Ability to measure (K+) currents in single cells

Disadvantages
- Difficult to apply in 3D constructs
- Automatization leads to a loss of accuracy
- Low throughput

Impedance measurements

Advantages
- Long-term effects can be measured

Disadvantages
- Responses cannot be measured at cellular level

Optical techniques

Advantages
- High throughput
- Can be automated
- Feasible in 3D constructs

Disadvantages
- Only relative values can be assessed, no absolute values

Figure 3. Methods to measure arrhythmogenicity. (A) Schematic overview of different methods to assess cardiomyocyte arrhythmogenicity. (B) Brief description of advantages and disadvantages of the respective methods.

parameters with regard to drug-induced anomalies. In particular, field potential duration is correlated with the APD. However, the correlation between field potentials and the underlying action potentials are complex,[138] and deduction of detailed information is difficult.[139] Cell density also influences multielectrode array recordings.[140]

Patch Clamp Analysis

With patch clamp analysis, ion currents can be measured.[141] Patch clamp analysis has already been performed in stem cell–derived cardiomyocytes in 2011,[142] with the first time recording of "a drug-induced modulation of cardiac action potentials in an automated patch clamp system," indicating that patch clamp analysis can serve as an automated method to predict cardiotoxicity. Patch clamp recordings in disease-specific hiPSC-CMs were able to reveal arrhythmias and drug effects.[143] Importantly, patch clamp techniques can be used to measure the repolarization of K+ currents in single cells, associated with TdP.[73] As previously mentioned, the great disadvantage of using patch clamp analysis is that this technique still remains a complex,

technically challenging procedure with a low throughput and the abovementioned automatization results in loss of accuracy.[141] It is also difficult to apply in 3D tissue, resulting in the need to isolate cardiomyocytes.

Impedance Measurements

Platforms to measure impedance contain electrodes incorporated into the tissue culture plates. A weak alternating current is applied between the electrodes.[144] Impedance is calculated as $Z = V/I$, and impedance measurements on hiPSC-CMs[136,145] capitalize on the mechanical movement of the beating cells. In 2011, Xi et al[146] described the xCELLigence RTCA Cardio System, which was specifically designed to measure impedance in hiPSC-CMs. Furthermore, impedance measurements are used to assess proliferation, viability, and cell-cell contacts,[141] which might allow their use in the assessment of structural toxicity. A disadvantage of impedance measurements is that without the ability to measure the electrical activity of cardiomyocytes, the drug response at a cellular level cannot be determined.[145]

Optical Techniques

Optical techniques can be used to measure increase in intracellular calcium during systole or membrane potential changes. Detection works via the change in the fluorescence intensity or emission spectrum. The method can be applied in an automated manner in stem cell–derived cardiomyocytes[147] and can also be used in perfused whole hearts.[148,149] The respective calcium/voltage-sensing dyes can be externally introduced, or stably expressed fluorescent proteins in the targeted cells can be used.[109,150] An example of a fluorescent protein is ArcLight, which can be used to measure the APD. ArcLight allows for the assessment of long-term drug effects, including proarrhythmogenic effects by studying APD.[109] However, when introducing the fluorescent proteins to the target cells, the electrophysical properties of the hiPSC-CMs can be altered, potentially biasing the results.[145] Multicellular 3D tissues have already been investigated in a high-throughput, high-content way with a system called OptoDyCE based on hiPSC-CMs, which combines the optical sensing of voltage and intracellular calcium or contractility.[111,151] An inherent disadvantage is the impossibility to measure absolute membrane potential values, as only relative values are assessable.[145] For a reliable assessment of proarrhythmic and antiarrhythmic effects, various assays can be combined,[152] as is also done by the CiPA initiative.[153–155]

Future Perspectives

Cardiac safety assessment has been troubled a long time by unreliable and/or technically challenging models, which were further limited by ethical concerns. Pluripotent stem cell-derived cardiomyocytes now offer opportunities to develop more reliable, but also simpler assays to measure cardiotoxicity which could eventually also replace animal experiments. We are currently in a transition phase, in which studies to validate stem cell-based assays are under way.[105] In a best-case scenario these assays complement each other to eventually allow safety screenings for arrhythmogenic, but also contractile cardiotoxicity. Further advantages could be the use of diseased induced pluripotent stem cell lines. For example, with clobutinol, one could consider the use of induced pluripotent stem cell lines that model long QT syndromes. In addition,

induced pluripotent stem cell lines derived from patients (or genetically modified) with dilatative cardiomyopathy might allow for investigating drug effects on heart muscle tissue with reduced contractile function. However, in contrast to the aforementioned applications, these ideas are farther away from a direct clinical/industrial application. Even farther away, nevertheless interesting, are considerations to use induced pluripotent stem cells to assess the individual risk (eg, to screen patients before starting an immunosuppressive or anticancer therapy).[113] Overall, stem cell–based strategies will change and improve the way cardiotoxicity screening is performed. Although animal models will remain indispensable for years, stem cell–based strategies offer the possibility to ultimately dispose of ethical concerns and species differences and thus increase throughput and predictive value. Stem cell–based cardiotoxicity screening is an exciting and evolving field that will benefit both individual patients and drug discovery pipelines in general.

DISCLOSURES

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