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*Modest.

†Significant.

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# Use of Human Induced Pluripotent Stem Cell–Derived Cardiomyocytes in Preclinical Cancer Drug Cardiotoxicity Testing:

## A Scientific Statement From the American Heart Association

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## Abstract

It is now well recognized that many lifesaving oncology drugs may adversely affect the heart and cardiovascular system, including causing irreversible cardiac injury that can result in reduced quality of life. These effects, which may manifest in the short term or long term, are mechanistically not well understood. Research is hampered by the reliance on whole-animal models of cardiotoxicity that may fail to reflect the fundamental biology or cardiotoxic responses of the human myocardium. The emergence of human induced pluripotent stem cell–derived cardiomyocytes as an in vitro research tool holds great promise for understanding drug-induced

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cardiotoxicity of oncological drugs that may manifest as contractile and electrophysiological dysfunction, as well as structural abnormalities, making it possible to deliver novel drugs free from cardiac liabilities and guide personalized therapy. This article briefly reviews the challenges of cardio-oncology, the strengths and limitations of using human induced pluripotent stem cell–derived cardiomyocytes to represent clinical findings in the nonclinical research space, and future directions for their further use.

**Keywords**

AHA Scientific Statements; biomarkers; cardiotoxicity; electrophysiology; human stem cell-derived cardiomyocytes; myocardial contraction; wounds and injuries

In the United States and Europe, cancer is the second leading cause of death and morbidity after cardiovascular disease (CVD).[1] On a global scale, cancer accounted for 9.6 million deaths in 2018.[2] Fortunately, present-day cancer treatment strategies have resulted in dramatic improvement in the survival of patients diagnosed with cancer. In 2016, there were an estimated 15.5 million cancer survivors in the United States, and this number is expected to increase to 20.3 million by 2026.[3] However, such gains in both quantity and quality of life are at risk of being partially erased by the increased mortality and morbidity from therapy-related CVD side effects.[4] The incidence of all cancers increases with age, which increases the number of cancer survivors who may have concomitant risk factors for cardiac disease at the time of diagnosis.[5] The use of cytotoxic chemotherapy is rising as cancer survival is improving.[6] Together, these factors mean that cancer is becoming a chronic illness, which creates an ever-pressing need to address both short- and long-term cardiotoxic effects of cancer therapy.

Cardiovascular complications of cancer therapy significantly contribute to the global burden of CVD, and the success in treating cancer has produced a large cohort of cancer survivors with increased risk of chronic multisystemic diseases.[7] Life-threatening complications of congestive heart failure, thrombogenesis, pericardial disease, hypertension, myocardial ischemia, cardiac arrhythmias, and vasospasm[8,9] have all been linked to cancer therapies such as cytotoxic chemotherapies, molecularly targeted therapies, and mediastinal irradiation, resulting in cardiomyocyte and vascular damage.[10] Cancer survivors may have markedly reduced life expectancy with evidence of coronary artery disease in their 30s, especially if additional risk factors such as hypertension, diabetes mellitus, obesity, and dyslipidemia are also present.[11,12] In particular, congestive heart failure as a result of cancer therapy has been linked to a 3.5-fold increased mortality risk compared with idiopathic cardiomyopathy.[13] The long-term risk of death resulting from CVD may exceed the risk of recurrence for many forms of cancer.[14,15] Therefore, as the population of cancer survivors grows, it is essential to recognize the need for early assessment of potential risks of acute and chronic cardiotoxicity. To that end, a better understanding of the mechanisms of cancer therapy–related cardiac toxicity is important to develop effective preventive approaches such as novel preclinical testing tools and methods that can assess the safety and efficacy of currently available and future therapies.

The focus of this scientific statement is the utility and potential promise of human induced pluripotent stem cell (hiPSC)–derived cardiomyocytes (hiPSC-CMs) in preclinical testing of drug-induced cardiotoxicity. Although there is overlap, it is useful to consider cardiotoxicity in 3 categories: electrophysiological abnormalities, contractile dysfunction, and structural toxicity. Although touching on all 3 categories, this review emphasizes toxicity related to myocardial injury with the use of in vitro hiPSC-CM preparations. Contractile dysfunction, usually the first clinical manifestation of drug-induced cardiotoxicity, is often associated with structural cardiotoxicity. The detection of structural cardiotoxicity with hiPSC-CMs in vitro may guide the evaluation of cardiac liabilities, the synthesis of safer molecules, and the design of more informative early clinical studies. The basic aspects of electrophysiological (proarrhythmia) toxicity and the ability of hiPSC-CMs to detect such effects, specifically those related to delayed repolarization and torsade de pointes (TdP), have been discussed extensively under the ongoing Cardiac In Vitro Proarrhythmia Assessment initiative[16,17] and are discussed briefly here.

It is recognized that animal models may not accurately represent human cardiotoxicity because of species differences in cardiac structure and function. For example, the rapid resting heart rate in rats and mice (300–400 and 500–700 bpm, respectively) necessitates different electrophysiological and calcium-handling systems compared with human cardiomyocytes. Other noted differences are related to mitochondrial content and metabolism.[18,19] The utility of nonhuman in vitro models to study cardiotoxicity depends on the recapitulation of mechanisms responsible for cardiotoxicity in the species substituting for humans. A similar situation is evolving with hiPSC-CMs, the key difference being that human clinical findings are being compared with those obtained from human-derived cell preparations that recapitulate the same physiology or pharmacological responses to various degrees. Various human-relevant hiPSC-CM–based models useful for cardiotoxicity testing are listed in Table 1.

Because the in vitro human-derived preparations are removed from any hemodynamic or neurohumoral influences, the contribution of such “indirect” drug effects on the larger cardiovascular system may be missed. For example, hypertension is a recognized hallmark of vascular endothelial growth factor inhibitors (and is sometimes considered a surrogate marker for target engagement) that could impose additional stress on the myocardium. hiPSC–derived endothelial cells can also be used to study vascular dysfunction (eg, tyrosine kinase inhibitor–induced vascular injury).[20] However, the complex integrated response involving multiple cellular vascular compartments hinders the ability to use stem cell–derived cell components to study the wide spectrum of drug-induced vascular toxicities (including accelerated atherosclerosis and thrombosis, endothelial dysfunction, peripheral artery disease, systemic hypertension, and pulmonary artery hypertension[21]) linked to oncology drugs. Any maladaptive cardiac response that may influence cardiac toxicity in vivo such as hypertension would not be included in in vitro studies. In addition, vascular toxicities resulting from drug effects on vascular structure and function would not be detected. However, direct effects of drugs on myocytes leading to cardiotoxicity would be present if the systems responsible for these responses are recapitulated in the human-derived preparations. This (along with a renewable source of a human-derived preparation) represents a key strength of using hiPSC-CM preparations in various evolving formats.

## CANCER GENETICS AND CARDIOTOXICITY OF CANCER DRUGS

A key objective of pharmacological oncology therapy is to retard the growth and metastasis of cancerous cells with minimal effect on normal cells. This objective requires specificity for targets that are expressed in cancer cells but absent in normal cells, as well as minimal off-target effects in normal cells. Such targeted drug activity is rarely absolute as a result of the presence of receptors and interconnected pathways shared by tumor tissues and critical organs, including the heart and vascular system.

It is useful to consider oncological drugs on the basis of 3 therapeutic approaches, namely conventional chemotherapy (as exemplified by the anthracycline doxorubicin), molecularly targeted therapy (as exemplified by tyrosine kinase inhibitors such as sunitinib and sorafinib), and immunotherapy (including checkpoint inhibitors and chimeric antigen receptor T-cell therapy).[22] In general, conventional chemotherapies are cytotoxins acting on all metabolically active cells, preferentially affecting (killing) rapidly dividing or metabolically overactive tumor cells. Thus, cytotoxins are generally less specific than molecularly targeted therapies and more likely to affect noncancerous cells. The heart is especially at risk of chemotherapeutic toxicity because cardiomyocytes have high metabolic rates, an increased presence of apoptotic machinery, and low regenerative capacity, all resulting in generally enhanced sensitivity to cytotoxic agents and a limited ability to cope with serious injury.

As an example, doxorubicin is associated with multiple direct cardiotoxic mechanisms, including production of reactive oxygen species, eliciting double-strand DNA breaks and apoptosis (via binding to DNA and topoisomerase II), impaired mitochondrial function and biogenesis, impaired sarcomeric maintenance, altered fatty acid metabolism, and stem cell senescence.[23] Impairment of prosurvival signaling pathways via NRG1 (neuregulin 1) signaling inhibition likely contributes to the well-recognized toxicity when doxorubicin is combined with trastuzumab, a molecularly targeted humanized monoclonal antibody against the extracellular domain of HER2/ERBB2 (human epidermal growth factor receptor *2*/receptor tyrosine-protein kinase erbB-2), which is a transmembrane receptor with intracellular tyrosine kinase activity overexpressed in up to 25% of breast carcinomas. The cardiotoxic effects of this combination of cytotoxic and targeted therapies likely result from the (on-target) blocking by trastuzumab of the ERBB family of receptors involved in cardioprotection in the setting of various stressors (including anthracyclines), an effect not anticipated or appreciated in early studies with trastuzumab as a sole agent. In vitro combination drug studies using hiPSC-CMs should prove useful in identifying potentially adverse pharmacodynamic interactions without placing patients at undue risk. The use of transcriptional and phenotypic profiling should make it possible to identify cell-specific toxicities, to predict the effects of combination therapies, and to differentiate drugs according to therapeutic classes and across human populationbased models.[24,25]

In regard to molecularly targeted therapies, numerous overlapping metabolic and signaling pathways linked to cardiomyocyte functions and survival are shared with tumor biology targets. Tyrosine kinase inhibitors are examples of molecularly targeted therapies expected to demonstrate reduced cardiac toxicity compared with conventional chemotherapeutic

agents. However, kinase selectivity is often difficult to achieve because of overlapping kinase activities in cancerous cells and adult human myocardium. Dendrograms (graphical representations depicting selectivity profiles of small-molecule kinase inhibitors) that compare the potency of drug block of multiple kinases shed light on the lack of specificity of tyrosine kinase inhibitors. Further complexity is provided by multiple cell types (eg, cardiomyocytes, fibroblasts, and endothelial cells) present in native myocardium that may be differentially affected by oncological drugs[26,27] and differential kinase expression in normal versus failing human hearts (the latter possibly more closely resembling cancerous cells[28]). The clinical effects of an oncological drug (and any active metabolites that are likely not generated in vitro) will depend on the cellular uptake of drug, differences in types and levels of kinase expression across cell types, and potential cross talk between adversely affected cardiomyocytes and other cardiac cell types and those within the vasculature.

Other marketed targeted therapies include proteasome inhibitors (cellular complexes that break down proteins, including cardiac sarcomeric proteins) and histone deacetylase inhibitors (affecting gene expression). Although the ability to evaluate responses of hiPSC-CMs for days to weeks proves useful for discerning direct cardiotoxic effects with these drug classes, the epigenetic background of hiPSC-CMs may confound responses to histone deacetylase inhibitors. Specific targeting of drugs may additionally involve more sophisticated drug delivery approaches, as with monoclonal antibodies and antibody-drug conjugates with unique antibody targeting or that overexpress cancerous cell surface receptors acting as drug delivery agents for oncologically toxic “payloads.” In some cases, screening for acute cardiotoxicity of antibody-drug conjugates and their various components (warhead, linker, and ligand portions) with hiPSC-CMs is already part of cardiac safety studies. Drugs tested on hiPSC-CM preparations have taken various forms, including small molecules, monoclonal antibodies, and antisense oligonucleotides. Studies are needed to establish the suitability of cultured hiPSC-CMs to test for cardiotoxicity with novel chimeric antigen receptor T cell–based therapies. As with all in vitro cell-based assay, challenges encountered with testing include delivering the drug at the specified (targeted) concentration, drug distribution within the test chamber, time course of exposure, presence (or absence) of metabolites that may be present in vivo, and extracellular accumulation of cellular products arising from the drug response.

## HUMAN IPSC-CM–DERIVED IN VITRO MODELS OF CARDIOTOXICITY

The Nobel Prize–winning invention of hiPSCs by Dr Shinya Yamanaka in 2007[29] has opened new avenues for drug screening, disease modeling, and ultimately personalized chemotherapy screening. With hiPSC-CMs, it is now possible to generate a virtually unlimited supply of any cell type based on differentiation protocols.[30] Thus, production of hiPSC-CMs is possible on a large scale with bulk purification and generation for rigorous cardiotoxicity testing in vitro[31,32] and on a smaller scale for patient-specific testing. These technologies enable bioengineering of cardiac tissue constructs of differing genetic and compositional complexities in 2 and 3 dimensions,[33] some of which may more closely resemble native adult cardiac tissues.

hiPSC-CMs are authentic cardiomyocytes in many respects, including expression of multiple molecular cardiac markers, action potentials, calcium transients linked to contractions, and, in particular, regular spontaneous beating, which helped in their initial identification and characterization. However, they also diverge significantly from adult cells, most notably in their disorganized structure, partially depolarized resting membrane potential, and spontaneous beating activity; the latter characteristic is not shared with either native adult atrial or ventricular cardiomyocytes. Despite these differences, which lead to their being labeled immature, hiPSC-CMs have attracted significant attention as experimental models because they are human derived, are readily available, and can be maintained in vitro for weeks to months. These characteristics are in contrast to isolated adult ventricular myocytes that rapidly dedifferentiate after a few days in culture. Thus, spontaneously stable beating hiPSC-CMs provide an exciting opportunity for cardiotoxicity studies lasting days to weeks (and possibly months) and are more amenable to genetic manipulation by transfection or gene editing. However, it should be noted that currently available hiPSC-CMs also demonstrate phenotypic plasticity during their in vitro life span, displaying (to varying degrees) different phenotypes (morphology, expression patterns, physiology, pharmacological sensitivity) in response to environmental clues, cellular companions, and (to some extent) time in culture.[34] Thus, it is important to clearly define environmental and experimental conditions (that may be specifically designed and selected) and the time window used in studies.

The immaturity of newly differentiated hiPSC-CMs is reflected in their transcriptome that most closely matches that of a first-trimester fetus.[35] For in vivo implantation, the immature characteristics of hypoxia resistance and residual proliferative activity may prove advantageous. However, they become disadvantages when in vitro model systems are expected to resemble adult human cardiomyocytes in form and function. Because of this, efforts are ongoing to push these cells toward a more mature phenotype. The main markers of success include structural changes (higher anisotropic ratio, well-developed and aligned sarcomeres, abundant mitochondria, and appearance of regular t tubules); switching to mature cardiac protein isoforms of troponin I (TNNI3 [troponin type I3]), myosin heavy chain (MYH6 [myosin heavy chain 6]>MYH7 [myosin heavy chain 7]), and $\beta_1$AR ($\beta_1$-adrenoceptor)>$\beta_2$AR ($\beta_2$-adrenoceptor); an action potential appropriate for the chamber type (eg, for ventricular: fast upstroke, notch, long plateau, stable resting potential at ≈−80 mV, no spontaneous pacemaker activity); increases in $I_{Na}$ and $I_{K1}$ currents; accelerated time to peak calcium/contraction and accelerated time to 50% relaxation; and decreased reliance on glycolytic metabolism.[36–40] These aspects of the contractile and electric maturity are necessary to recapitulate as closely as possible the adult myocyte for the evaluation of contractile dysfunction. However, it should be noted that even immature hiPSC-CMs are useful in detecting various forms of drug-induced cardiotoxicity when the systems affected by drugs are present (eg, negative inotropic agents). In particular, the ability of hiPSC-CMs to detect drug-induced surrogate markers of TdP proarrhythmia (sometimes referred to as “cellrhythmias” and including early afterdepolarizations, abnormal depolarizing waveforms occurring during ventricular repolarization) and triggered activity (an early afterdepolarization that reaches threshold to elicit an all-or-none depolarization event) has been clearly demonstrated by the Cardiac In Vitro Proarrhythmia Assessment initiative and

related studies as a result of the presence of key ionic currents that define such cellular proarrhythmic effects.[16] The extent to which a more mature phenotype may affect the sensitivity and fidelity of translation of hiPSC-CMs as proarrhythmic models remains to be determined.

Some improvements in the maturity of hiPSC-CM phenotypes are evident by extending the time of culture, although even periods up to 9 months may not fully reproduce the adult phenotype.[34,41] This is challenging for high-throughput investigative studies, and accelerating maturation strategies are frequently reported in the literature. Maturation-linked hormones and growth factors (eg, thyroid hormone [$T_3$], corticosteroids,[42,43] and selected microRNAs[44]) have produced significant improvements in phenotype. Many of the methods for improving maturity concentrate on re-creating the mechanical and electric stimuli that the cell would have experienced if integrated within an adult heart. Anisotropic substrate patterning[45] or optimized extracellular matrix[46,47] can improve calcium handling in 2-dimensional (2D) cultures. The term human-relevant preparations has been used to describe the multiple emerging formats of in vitro preparations using hiPSC-CMs to create more complex cardiac structures as the source of myocytes, moving beyond 2D monolayers to contractile sheets, 3-dimensional (3D) constructs, and engineered human tissues.

A number of technology platforms integrating 3D myocyte constructs have been developed with the use of hiPSC-CMs, which are often encased in a hydrogel substrate that condenses with time to bring the hiPSC-CMs into closer contact.[36,38,48,49] After a few days, hiPSC-CMs form connections and beat as a syncytium; suspending the spontaneously beating constructs between supports allows them to perform work and to develop further.[48,49] Manipulating the preload and afterload can accelerate this and, in the extreme, mimic the overload, leading to manifestations of pathological hypertrophy.[40,50] Intensive and sustained pacing regimens to increase beating rates up to 4 Hz have also been added to further increase the physical load on 3D engineered heart tissue.[39] Positive Frank-Starling and force-frequency relationships were produced by this method, as well as a highly developed muscle structure.

Engineered 3D constructs have successfully produced cells with a high anisotropic ratio, accelerated calcium transient, greater sarcomeric density, and a rudimentary t-tubule system. However, force generation is a key output to judge the convergence to the adult phenotype, and the intensive pacing method (with forces of ≈6 mN/mm$^2$) was not as effective as dynamic perfusion culture[37] (with reported output of up to 30 mN/mm$^2$); this compares with 25 to 44 mN/mm$^2$ for adult human trabeculae.[51,52] Transcriptome data have also confirmed the improved maturation, with more adult-like conduction profiles (increased KCHN2 and decreased HCN4), sarcomere- and t-tubule–associated proteins (MYH7, TNNI3, JPH2 [junctophilin 2], CAV3 [caveolin 3], and BIN1 [bridging integrator 1]), and key calcium-handling components (ATP2A2 [ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2] and RYR2 [ryanodine receptor 2]).[39] However, even with automated measurement systems[53] or downscaling to microtissues,[54] these 3D constructs represent a reduction in throughput compared with monolayer preparations, which use less direct (or surrogate) measures of contractile force.

Certain aspects of immaturity have proved more challenging than this natural maturation strategy can accomplish, including the abolition of the pacemaker potential and appearance of a fully developed t-tubule system. Although the abolition of intrinsic pacemaking activity is an electrophysiological sign of cardiomyocyte maturity, the presence of pacemaker (nodal) cells in 2D constructs will always conveniently produce monolayers with spontaneous activity (thus avoiding the need for electric or optogenetic-based pacing). Ventricular t tubules, which are essential to fully recapitulate excitation-contraction coupling in ventricular myocytes and to provide for spatial control of βAR (β-adrenoceptor) subtype–dependent signaling,[55] remain a hallmark of overall hiPSC-CM maturity.

It is interesting to compare results obtained with in vitro maturation efforts with results obtained from in vivo implantation of hiPSC-CMs in which a (presumably) more complete cardiac environment would be expected to achieve maximum maturation. Here, t tubules ($CAV3^+$ invaginations) are not as extensive or defined as in the adult host cells even after several months, and there is evidence for continued automaticity of the graft[56] that is possibly related to an absolute limitation of hiPSC-CM maturation. For in vitro maturation, more aggressive techniques such as forced expression of Kir2.1 ($I_{K1}$) to hyperpolarize the membrane and to prevent phase 4 depolarization[57] or treatments that increase expression of t-tubule structural proteins[58] have made further inroads into phenotypic maturity.

In terms of the assessment of longer-term drug-induced cardiac toxicity for which the hiPSC-CMs may be particularly valuable, other aspects need to be considered. Adult cardiomyocytes have low proliferative activity but typically increase in size and sarcomeric organization in response to work stress. This can culminate in hypertrophy, which is a known pathophysiological marker. Hypertrophic responses of hiPSC-CMs to α-adrenoceptor stimulation (often used in animal studies) are not strong in terms of cell size increase in 2D culture, with hiPSC-CMs reported to be less responsive than human embryonic stem cell–derived CMs.[59] In these studies, brain natriuretic peptide release is often used as a surrogate readout with endothelin-1 as the agonist.[60] As noted, hypertrophy can be mimicked in 3D tissues by an increased workload, but even then the increase in cell size can be absent.[37] Despite some striking successes in modeling cardiotoxicity in terms of cell death response to various agents, there is no doubt that hiPSC-CMs are more robust than isolated adult cardiomyocytes, as shown by their long survival and resistance to hypoxia.

Although hiPSC-CMs target specific functional parameters, improvements in their maturity do not confirm the ability of either the simpler or the more mature hiPSC-CM preparations to respond to stress- or drug-induced injury in a manner similar to that of adult ventricular myocytes. Such confirmations will require studies demonstrating appropriate responses that compare nonclinical findings with clinical cardiotoxic experience. These confidence-building validation efforts also rely on clinical gold standards with which to compare consensus biomarkers. Recent efforts indicate that this is best accomplished by using more complex 3D constructs consisting of multiple cardiac cell types (eg, fibroblasts, endothelial cells, and myocytes) as in organoids, 3D constructs, or engineered heart tissues,[54,61,62] including heteropolar heart tissues with atrial and ventricular ends.[63] The requirements for consistent and reproducible complex cardiac preparations will provide an additional challenge for in vitro cardiotoxicity studies that must include adequate characterization and

descriptions of preparations, comprehensive standards for acceptable preparations, and consensus biomarkers.[60,64,65] The need for demonstrating "fit-for-purpose" applications is key because not all cardiotoxicities will likely be present in the various human-relevant preparations in use or being developed because of differences in myocyte maturity, the influence of additional nonmyocyte cardiac cells that may be affected by drugs and influence cardiomyocyte responses, or the plastic phenotype of hiPSC-CMs in culture over time. Finally, some cardiotoxic effects may not be detected even if perfectly mature complex cardiac preparations were available as a result of the influence of extracardiac signaling, drug-induced cardiovascular stresses (eg, hypertension), or direct influences of the targeted cancer cells on the heart or cardiovascular system.

## UTILITY OF HIPSC-CMs FOR DEFINING ACUTE VERSUS CHRONIC CARDIOTOXICITY

Cancer is being transformed from a rapidly fatal disease to a chronic condition in part as a result of more efficacious oncological therapies. Such success has heightened awareness of longer-term cardiotoxic effects that may manifest weeks to months (or longer) after discontinuation of therapy. For example, anthracycline-induced cardiotoxicity can occur in the short term (within days of administration) or can occur within 30 days or even as long as ≥10 years after a single administration. Indeed, delayed-onset cardiotoxicity may take longer to progress (or detect) than typical toxicological studies used to detect such effects in animal models.

Clinically, cardiotoxicity has traditionally been characterized as acute, subacute, or chronic.[66] Acute toxicity (defined as arrhythmias, electrocardiographic changes, pericarditis, or myocarditis and heart failure) is typically rare early during therapy compared with the more frequent subacute cardiotoxicity (within 1 year of therapy) and chronic toxicity, which have incidences reported in the literature varying from 1.6% to 23%.[67] Late-onset cardiotoxicity may occur 10 to 20 years after drug administration and is likely related to newly imposed stress on a previously injured or damaged cardiac substrate. Whatever the time course of development, a rigorous, systematic longitudinal analysis of clinical cardiotoxicity of marketed oncological drugs is needed. The resultant data will inform patient care while serving to benchmark the utility of hiPSC-CM models and biomarkers for the early detection of cardiac liabilities.

Adult human cardiac tissue and cardiomyocytes are largely unavailable for longer-term in vitro studies and cannot be maintained in the laboratory with standard tissue culture approaches because of the rapid dedifferentiation observed in typical culture conditions. Consequently, only a minimal number of in vitro studies evaluating the short-term toxic effects of drugs are possible with isolated human ventricular myocyte preparations obtained from a few select myocyte sources. Furthermore, the consistency and reproducibility of responses from such limited sources and studies may prove problematic. In contrast, hiPSC-CMs are readily available, making them especially attractive for the study of short- and longer-term cellular responses, as in the case of oncological drugs.[68–70] It is anticipated that early markers of cardiac injury (some recognized clinically) may be useful to detect and

predict longer-term clinical cardiotoxicity. In cases when there is discordance, further studies are necessary to determine (1) whether hiPSC-CMs were somehow deficient in demonstrating a response, (2) whether cardiotoxicity required the involvement of other cardiac cell types (eg, fibroblasts, endothelial cells, or atrial cells), (3) whether cardiotoxicity required additional systemic influences not present in in vitro studies (renal or hepatic signaling, cardiotoxic metabolites, etc), or (4) whether there was inadequate matching of in vitro exposures with clinical exposures. Arguably, the in vitro demonstration of cardiotoxicity with an evolving drug candidate is a form of hypothesis generating, with corroboration provided (or required) from either traditional toxicological studies or slow and deliberate dose-escalating clinical studies (if the cardiotoxicity can be monitored or is reversible). This approach would avoid potential false-positive safety findings with hiPSC-CMs that might lead to the unwarranted attrition of lifesaving drugs. For these reasons, the ability to test for direct cardiotoxic effects of novel drug candidates early in the drug discovery process (thus influencing compound selection) while reducing the reliance on animal studies is clearly advantageous and is presently being further evaluated and calibrated.

The in vitro detection of longer-term clinical toxicity that develops over months to years will likely have to rely on early markers of injury or cellular stress.[71] Although it is possible to maintain hiPSC-CMs for months in culture where they may continue to beat spontaneously with stable baseline function,[72,73] it is not realistic to routinely conduct in vitro studies extending from months to years. Instead, it is more practical to rely on more traditional in vivo nonhuman toxicological studies. In cases when a drug may suppress spontaneous automaticity of hiPSC-CM preparations, external stimulation, either electrically or with optogenetic approaches, is required. It is likely that drug effects on spontaneous beating rate may influence the development of cardiotoxicity via changes in the metabolic demands and calcium handling of myocytes, necessitating consideration of long-term paced myocyte studies, an approach that would require refinement to ensure that the pacing techniques themselves did not influence cardiotoxicity.

Two in vitro studies with doxorubicin demonstrate the ability to detect cardiotoxic effects with hiPSC-CMs. Louisse et al[20] studied the effects of low-dose doxorubicin (150 or 300 nmol/L) on hiPSC-CMs using a single-dose (up to 2 days) and multiple-dose administration (repetitive dosing over 14 days), with phenotypic assays done on day 14. They found significant mitochondrial dysfunction, including depolarized membrane potentials and elevated calcium levels, after 2 weeks of dosing. This comprehensive in vitro examination of the short-term and longer-term effects of a drug on human cardiac phenotypes with inclusion of overall membrane electrophysiology and subcellular-mitochondrial function is made possible with hiPSC-CMs. More recently, Holmgren et al[68] used human embryonic stem cell–derived CMs from a commercial source combined with “omics” data (protein, mRNA, and microRNA) to identify differential expression in response to doxorubicin. Their dosing included a 2-day exposure followed by a 12-day washout period to identify early biomarkers of cardiotoxicity. In addition to phenotypic effects on cell morphology and function, the data showed a strong effect of doxorubicin on all molecular levels tested. These results identified multiple mechanisms of cardiotoxicity and suggested putative biomarkers for chemotherapy-induced cardiotoxicity.

## ESTABLISHING STANDARDS AND PURSUING REPRODUCIBILITY

One of the greatest challenges in the reproducibility of hiPSC-CM cardiotoxicity testing is generating cardiomyocytes of homogeneous phenotypes across multiple laboratories.[74] This challenge currently is mitigated by the use of cells produced by 2 major commercial vendors, although functional variations between suppliers and batch variations have been noted.[75] Although the use of commercially available cells promotes reproducibility for a cell line, costs may hamper large-scale experiments. It is recognized that the reliance on a few commercial myocyte sources (each producing a myocyte derived from 1 donor) contributes to known differences in functional baseline characteristics and pharmacological responses across 2D myocyte preparations[75] and ultimately prevents studies investigating the heterogeneity of responses across a diverse population of hiPSC-CMs. It has been demonstrated that hiPSC-CMs generated with diverse differentiation protocols, along with those produced by commercial vendors, have generally similar phenotypes[76,77] and ratios of ventricular, atrial, and nodal subtypes,[78] but functional variation exists. The generation of homogeneous chamber-specific preparations (to be used either alone for studies or after combination with other cardiac-derived cell types) remains an aspirational goal despite recent progress in the control subtype specification.[79] Finding the optimal mix of atrial, nodal, and (predominantly) ventricular electrophysiological phenotypes in both academic and commercial preparations has also proved problematic, especially for studies of drugs affecting atrial fibrillation.

Substantial efforts have been made to enhance the reproducibility of cardiomyocyte differentiation protocols with the assumption that simplicity, ease of use, and cost-effectiveness will drive universal application. This has resulted in common protocols now simplified for the use of small molecules such as the GSK3B (glycogen synthase kinase 3-β) inhibitor CHIR99021 and WNT inhibitor Wnt-C59 to modulate WNT signaling to drive cardiac differentiation,[80] as well as the application of very simple media formulas containing just basal medium, albumin, and ascorbic acid.[81] The adoption of a single standard differentiation protocol would aid cross-laboratory cardiotoxicity comparisons but may be difficult to assess because of the myriad cell and handling factors involved.

Most cardiomyocyte differentiation protocols produce cells of ≈70% to 90% purity, commonly assessed by flow cytometry for troponin T (TNNT2) with specific antibodies (clone 13–11).[82] Many laboratories are interested in enhancing cardiomyocyte purity to further reduce experimental variability. Simple glucose starvation methods[31] have not proven efficient, but antibiotic resistance cassettes such as *MYH6*-puro,[83] which are commonly used in commercial cells, have proved effective. In addition to purity, the desire for more mature cardiomyocytes has complicated some protocols, particularly in the use of the metabolic maturation media that replaces glucose with palmitate,[84] which is known to modulate toxicity assays.[85] Existing glycolytic conditions that contain 11 mmol/L D-glucose (315 mg/dL, similar to that found in patients with hyperglycemia) are arguably more akin to those found in the hypertrophied heart and may have a negative impact on drug testing assays. A wide range of metrics have been identified as part of a multiparametric quality assessment rubric that combines structural, electrophysiological, and contractile measurements to characterize cardiomyocyte preparations and their maturation status.[86,87]

If not adequately controlled, factors such as cell handling, timing, and culture medium used during drug dosing can contribute prominently to overall response variability and reproducibility. This aspect has been understudied, particularly in regard to potential sensitivity to drug-induced cardiotoxicity. It highlights the need for inclusion of positive and negative controls in studies to calibrate assay sensitivity and specificity and, from a drug development perspective, to prevent false-positive results, which cause needless and wasteful attrition of novel therapeutics. This issue will become increasingly important as newer and more complex preparations developed to provide more mature phenotypes[88] are introduced.

In summary, much of the progress toward establishing standardized and reproducible cardiotoxicity analysis relies on investigators being aware of new developments in the field, actively adopting advanced techniques, and providing information characterizing the myriad preparations under study. Providing sufficient information to reproducibly generate hiPSC-CMs, in the case of noncommercial sources, and to reproducibly generate hiPSC-CM preparations of different levels of complexity and maturation, in the case of functional studies, is a first step toward enabling reproducible assessment of cardiotoxicity with human-relevant preparations in vitro. Along with the use of standardized protocols, this will enable a best practice for cardiotoxicity assays to be developed with universal applications.

## IN VITRO APPROACHES TO ASSESS CARDIOTOXICITY: TECHNIQUES, BIOMARKERS, AND RELATION TO CLINICAL BIOMARKERS

A biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention. A preclinical biomarker may be judged useful if it is linked to known mechanisms responsible for cardiotoxicity or is predictive of clinical cardiotoxicity of uncertain causes. Biomarkers may serve as useful tools for hazard identification or, in the case of more developed or qualified biomarkers, for risk assessment. When qualified, a biomarker is linked to a specific interpretation and application for drug discovery or regulatory purposes within a stated context of use.

Evaluating concordance between preclinical and clinical cardiotoxicity biomarkers is made difficult by variability in detecting and defining subclinical and clinical contractile dysfunction, which is often the first sign of evolving cardiotoxicity. Furthermore, nonclinical challenges are created by the electrophysiological and contractile immaturity of hiPSC-CMs, as well as the simplified cardiac substrate in vitro (specifically, cardiomyocytes alone without supporting fibroblasts, endothelial cells, and neurohumoral influences present in vivo). Although the multiple overlapping pathways within hiPSC-CMs and adult myocytes provide confidence in the ability to recapitulate the mechanisms responsible for cardiotoxicity and to detect potential safety liabilities, it is essential to demonstrate appropriate responses in vitro to define the utility of the in vitro hiPSC-CM models.

Challenges are also presented by the time course for the development of cardiotoxicity, with some drugs such as doxorubicin manifesting clinical toxicity of various degrees months to years after dosing. Although it may be impossible to detect such long-term effects in vitro,

one might be able to detect subtle signs of injury early through early and sensitive upstream biomarkers such as transcriptional changes, cardiac troponins, brain natriuretic peptide, microRNAs, or vesicles.[89] Such studies may provide insights into direct cardiotoxic mechanisms and novel biomarkers for in vitro or subsequent clinical applications. Studies comparing biomarkers of myocardial injury–structural cardiotoxicity with general cytotoxicity studies (eg, human stem cell–derived neuronal or hepatic preparations) would be useful for elucidating general cytotoxic effects versus specific cardiotoxic effects of agents. Of course, any hemodynamically driven effects or indirect effects requiring multiorgan integrated systems-based interactions will not be detected by either simpler (2D) or more complex in vitro tissue preparations, similar to toxicities involving immunological component (eg, myeloperoxidase released from neutrophils).

Molecular and cellular mechanisms of cardiotoxicity are diverse and complex, reflecting the complex interactions of on-target and off-target drug effects, diverse pathways, and underlying pathologies of the heart and cardiovascular system.[90] As a result of our lack of understanding of multiple potential mechanisms, a phenotype-based approach using hiPSC-CMs may be better suited to detect potential cardiotoxicity hazards, providing a more comprehensive and species-specific perspective for developing novel oncological drugs. Clearly, preclinical and clinical efforts are complementary and necessary to define biomarker concordance in the future. Table 2 provides a brief overview of the different technologies and biomarkers, along with associated advantages and disadvantages, used to assess electric, contractile, and structural cardiotoxicity with hiPSC-CMs.

## Biomarkers of Electric Cardiotoxicity

Delayed repolarization, which is manifest as prolongation of the QTc on an ECG, represents a surrogate biomarker of TdP, a rare polymorphic ventricular tachycardia characterized by “twisting of the points” morphology that may degenerate to ventricular fibrillation. A number of oncology drugs are associated with QT prolongation, a surrogate marker of proarrhythmia,[91] although the incidence of TdP is rare.[92] Despite the putative electrophysiological immaturity of 2D sheets of hiPSC-CMs, recent efforts have demonstrated their ability to detect concentration-dependent delayed repolarization using either extracellular field potential recordings or voltage-sensing dyes under the Cardiac In Vitro Proarrhythmia Assessment initiative.[16,17] As with in vivo studies, drug effects may include increases (decreases) in beat frequency that affect shortening (prolongation) of repolarization independently of the direct effects on repolarizing currents, necessitating the application of an appropriate in vitro beat rate correction formula[93,94] if pacing is not used. Further efforts are ongoing to characterize the electrophysiological effects of more complex cocultures and 3D tissue constructs, which may demonstrate altered sensitivities compared with simpler preparations. Future studies will define the role of hiPSC-CM–based preparations in characterizing the long-term effects of drugs on ionic currents such as those resulting from altered channel expression (eg, hERG [KCNJ2]) or trafficking, as well as cellular metabolism, stress, or cellular injury. Efforts to assess the potential risk of drugs affecting atrial fibrillation, which has been noted for some tyrosine kinase inhibitors, are beginning with the recent development of commercially available atrium-specific hiPSC-CMs.

## Biomarkers of Contractile Cardiotoxicity

Cardiotoxicity of oncological drugs is typically defined clinically by reductions in cardiac contractility, which is influenced by the inotropic status of the myocardium, preload, and afterload (the last 2 are not recapitulated in current hiPSC-CM platforms). Potential mechanisms responsible for short-term negative inotropic effects include dysregulated calcium handling and alterations in excitation-contraction coupling, whereas longer-term effects include functional and structural alterations in the contractile apparatus or myocyte injury that could potentially lead to myocyte loss via necrosis or apoptosis.

It is possible to study short-term inotropic effects with 2D hiPSC-CM contractions using optical approaches (eg, by monitoring edge movement, traction force, or intracellular calcium transients), electric approaches (eg, by using impedance-based approaches that provide indirect measures of myocyte movement or optical mapping of sarcolemmal membrane potential), or direct measures of force (eg, 2D films, cantilever systems, etc). Further studies are necessary to calibrate the short-term negative inotropic effects of oncological drugs for simpler 2D hiPSC-CM sheet preparations, as well as more advanced and complex 3D structures.[34,61] Early studies are ongoing to demonstrate the utility of hiPSC-CMs to detect longer-term effects (weeks to months) on contractile function, thus resembling a more traditional nonclinical toxicology study. The success of these in vitro studies depends on the extent of recapitulation of structural characteristics and functional (excitation-contraction coupling) mechanisms present in hiPSC-CMs compared with native ventricular myocytes of those systems affected by drugs.

## Biomarkers of Structural Cardiotoxicity

Cardiomyocyte injury and loss, along with accompanying fibrosis, contribute to short-term and long-term (and sometimes irreversible) cardiotoxicity. Biomarkers such as cardiac troponins, NT-proBNP (N-terminal pro-B-type natriuretic peptide), and myeloperoxidase have been shown to have a predictive role in the development of clinical cardiotoxicity with oncological drugs,[95] and integrating troponin levels with early reductions in global longitudinal strain may have incremental value in predicting future cardiotoxicity[96] and in guiding therapies. Various biomarkers such as cardiac troponins, heart fatty acid–binding proteins, microRNAs, and vesicles have been used preclinically to detect hiPSC-CM injury or death in vitro.[70] These biomarkers can be combined with functional and morphology-based studies to provide a more comprehensive in vitro assessment of structural cardiotoxicity.[97,98]

Although the triggers and mechanisms responsible for release of cardiac troponins from myocytes remain to be fully characterized, this biomarker continues to be actively pursued in nonclinical and clinical studies, partly because of its prior use as a clinical marker for ischemia/infarction and the development of ultrasensitive troponin assays. Ideally, biomarkers should be able to be used preclinically and clinically, with one use informing the other. It will be challenging to quantitatively compare in vitro biomarker responses with clinical responses because of the lack of standardization of in vitro preparations, experimental conditions, and differences in the disposition of secreted/excreted biomarkers in vitro versus in the human body. Further refinement of the functional characteristics of

hiPSC-CMs with the use of “organs-on-chips” approaches may help to clarify quantitative comparisons of in vitro and in vitro secreted or released biomarkers using human stem cell–derived preparations.[99]

In vitro–based biomarker studies may also prove useful in evaluating the safety of oncological drug combinations, providing the potential to use the same biomarkers to ensure safety in clinical use. Specific patient-derived myocytes may be useful in detecting individuals more sensitive to cardiotoxicity[69] (see also the Genome Editing section). Such approaches will require consistent and reproducible reprogramming, along with controlled differentiation strategies, to preserve interindividual variation in hiPSC-CMs without increasing in intraindividual variation, as judged by transcriptome profiling.[100]

## GENOME EDITING

Although patient-derived hiPSC-CMs have proven powerful for modeling monogenic diseases, the vast majority of CVDs are multifactorial, with complex genetic and environmental contributions.[64,74] Susceptibility to drug-induced cardiotoxicity in any given individual is also multifactorial. Some gene variants such as those found to be associated with drug-induced QT prolongation and TdP may increase sensitivity to drugs, whereas other gene variants may confer relative resistance to drug-induced cardiotoxicity.[101,102] To design hiPSC-CM models that more accurately reflect the genetic basis for differential drug sensitivities and to dissect the mechanisms of drug-induced toxicities, there is significant interest in using genomic-editing techniques to precisely modify the gene sequences in hiPSC-CMs suspected of enhancing the risk of cardiotoxicity.

Genome-editing techniques are broadly classified on the basis of the nucleases used to create sequence-specific double-stranded breaks: ZFNs (zinc finger nucleases), TALENs (transcription activator–like effector nucleases), and CRISPR (clustered regularly interspaced short palindromic repeats)–associated nuclease Cas9 (CRISPR/Cas9).[103] Double-stranded breaks are repaired in the cell by 2 distinct DNA repair mechanisms: nonhomologous end-joining or homology-directed repair. Nonhomologous end-joining repair typically results in insertions or deletions, which often generate loss-of-function mutations. In homology-directed repair, a single- or double-stranded DNA template is used to repair the break site. With the use of homology repair templates that carry a site-specific mutation, a desired sequence change can be introduced to any target gene in the cell. In contrast to ZFNs and TALENs, which target specific DNA sequences through a protein-DNA interaction, CRISPR-Cas9 is targeted to specific DNA sequences through a complementary base pairing involving a gRNA (guide RNA).[104] The relative ease of genome targeting with site-specific gRNA enables multiplexed gene editing,[105] allowing CRISPR-Cas9 to rapidly become the dominant technique for genome editing. Although early efforts in CRISPR-Cas9 genome editing resulted in low rates of homology-directed repair at target sites and the introduction of insertions or deletions at unintended sites, a number of innovations have led to substantial improvements in precise editing efficiencies and a reduction in off-target alterations.[106,107]

With genomic editing, hiPSC lines with genetic modifications that increase or decrease susceptibility to cardiotoxic drugs can be generated. For instance, CRISPR-Cas9–mediated disruption of *TOP2B*, which encodes Top2b (topoisomerase-II β), was used to demonstrate that hiPSC-CMs lacking this enzyme were resistant to doxorubicin-induced DNA damage and cell death.[102] This confirmed earlier mouse studies suggesting a role for topoisomerase-II β in doxorubicin-induced cardiotoxicity. Future applications of genomic editing might include developing a platform to assess the cardiotoxicity propensity of a drug candidate in a panel of hiPSC-CMs that reflects a range of susceptibilities. These efforts will overlap with those aimed at generating isogenic iPSC-CM models of cardiovascular diseases, such as those recently developed for myosin heavy chain,[33] myosin-binding protein C3,[108] and lamin A/C[109] cardiomyopathy using CRISPR/Cas9 editing approaches. In addition, different combinations of common variants that constitute the full range of a genetic QT risk score[101] can be engineered and deployed to generate a proarrhythmic risk score of drugs that more accurately assesses the degree of risk posed to the general population, beyond the extreme sensitivity of monogenic models. Finally, genome editing of hiPSCs can also be a valuable approach to delineate the pathogenicity of variants of uncertain significance in patients with suspected cardiomyopathy[110] and channelopathy.[111]

## APPLICATION OF IN SILICO AND SYSTEMS BIOLOGY APPROACHES TO HIPSC-CM MODELING

The enhanced sophistication of biological data resulting from computing power, analytical software, and sensing technologies makes it possible to conduct computational modeling of hiPSC-CM genomics, structure, and function in preclinical cancer drug cardiotoxicity testing. Several in silico models have already been developed as a good start. In a landmark article, Tentner et al[112] used computational modeling to understand signal transduction pathways after DNA damage by doxorubicin. Their results suggested a role for complex, cytokine-modulated interrelationships among multiple signaling pathways, including ERK (extracellular signal-regulated kinase), in both G1/S arrest and apoptotic cell death. More recently, Alkan et al[113] developed a multiscale computational model linking chemotherapy-induced DNA damage signaling to cell fate that was trained and calibrated for U2OS osteosarcoma cells.[114] Similar in silico models could be combined with results obtained with hiPSC-CMs to understand the molecular mechanisms of drug-induced cardiotoxicity. de Oliveira and Niederer[114] conducted in silico experiments to predict mechanisms of doxorubicin-induced mitochondrial dysfunction in hiPSC-CMs during the acute and chronic stages of cardiotoxicity. Their simulations predicted that direct mitochondrial DNA damage at therapeutic exposures is the principal pathway leading to progressive and irreversible long-term mitochondrial dysfunction and chronic cardiotoxicity. These are just 3 examples of potential applications of in silico and systems biology approaches that could guide similar efforts in hiPSC-CM biology to understand, predict, and avoid structural cardiotoxicity. The combination of hiPSC-CM research with in silico modeling may be extended to test different doxorubicin treatment protocols and combination therapies, patient-specific mechanisms of susceptibility (or resistance) to chemotherapy-induced cardiomyopathy, or pharmacologically based cardioprotection strategies.

Arguably, the ultimate goal of in silico approaches is to develop an effective platform for predicting potential emergent structural and functional cardiotoxic liabilities by integrating multiple experimental observations from hiPSC-CMs to inform in silico–based reconstructions. In this approach, candidate cardiotoxicity end points are measures of nuclear and mitochondrial DNA damage and apoptosis, as well as structural and electromechanical defects leading to arrhythmias and heart failure, as seen in early- and late-onset cardiotoxicity. Such in silico modeling approaches could enable detailed exploration of multiple candidate parameters associated with anticancer drug cardiotoxicity, explorations that would be impossible to achieve from characterization of drug effects on any given parameter at a time. As with any in silico modeling efforts, care should be taken because the lack of high-quality data with which to constrain the models hampers the ability to distinguish plausible from incorrect predictions. A recent comparison of hiPSC-CMs and adult native ventricular myocyte in silico models focusing on essential functional electrophysiology and calcium-handling features provides a useful starting point for further experimental characterization of evolving hiPSC-CM preparations for normal and disease modeling efforts. With the help of genomics, proteomics, and advances in hiPSC-CM maturation, mathematical models could provide valuable information to quantitatively evaluate and improve the utility of hiPSC-CMs for cardiotoxicity testing and human cardiac disease modeling.[115]

## PATIENT-DERIVED DISEASE MODELS: PERSONALIZED PREDICTIONS OF ONCOLOGY DRUG CARDIOTOXICITY

Although personalized medicine goals are typically focused on efficacy, comparable efforts to identify and avoid therapies that may lead to serious adverse on- or off-target cardiotoxic effects in specific patients are just being considered. Researchers are studying the use of hiPSC-CMs for defining cardiotoxicities on individual and population levels.[74] It has been demonstrated that hiPSC-CMs are capable of recapitulating the predilection of an individual patient with breast cancer to doxorubicin-induced cardiotoxicity, showing that hiPSC-CMs from patients with breast cancer who developed cardiotoxicity could recapitulate the increased risk in vitro.[69] In this model, 12 hiPSC lines were generated: 4 from patients who were treated with doxorubicin (240 mg/m$^2$) and experienced cardiotoxicity (ejection fraction, 10%–45%), 4 from patients who were treated with doxorubicin without cardiotoxicity, and 4 from healthy age- and sex-matched control lines. Lines from patients with cardiotoxicity demonstrated decreased cell viability, altered mitochondrial function and calcium handling, and increased reactive oxygen species production compared with patients who did not experience cardiotoxicity. These data demonstrate that hiPSC-CMs can be used to evaluate patient-specific differential responses to doxorubicin. More recently, a similar approach has also been demonstrated in patients with breast cancer who experienced cardiac dysfunction caused by treatment with trastuzumab (Herceptin).[116]

The successful application of hiPSC-CMs to patient-specific oncology drug-induced cardiotoxicity relies on the hypothesis that predisposition to cardiotoxicity is genomic (ie, translated to in vitro hiPSC-CM models); however, this commonly held belief has not been experimentally validated to any large extent. Many interpatient genomic variables might

influence how cardiomyocytes are affected by an oncology agent, including the effectiveness of their inward and outward transporters, the presence of metabolizing enzymes, and protective mechanisms against reactive oxygen species. Many of these factors may be modeled in silico or altered genetically. The contributions of environmental factors, epigenetics, and prior diseases (such as diabetes mellitus) to cardiotoxicity, as well as drug responses from nonmyocytes within the myocardium (eg, fibroblasts, endothelial cells) and the vascular compartment, remain to be determined.

Although the patient-specific hiPSC-CM model is simple in concept, a number of limitations are readily evident. For example, hiPSC-CMs do not have the high numbers of mitochondria seen in the adult heart described above. One advantage of hiPSC-CMs is that they are relatively pure single cells, which can also be considered a weakness because many cardiac and vascular cell types (as well as other organ systems) may be involved in cardiotoxic responses. For example, some single nucleotide polymorphisms that have been associated with cardiotoxicity are in genes expressed only in the liver. They likely affect drug metabolism and represent a major aspect of the challenges of translating cardiotoxicity from a simpler in vitro system.

Although the application of hiPSC-CMs for personalized predictions of cardiotoxicity is very much in the infancy stage, hiPSC-CMs provide a novel approach to individualized cardiotoxicity testing that may prove better at protecting patients and promoting future drug development efforts. In particular, efforts should be focused on investigating the relationship between the genome and the risk of cardiovascular toxicity and discovering analogous pathways for defining efficacious drugs for various tumors on the basis of sequencing data.[117]

Although not the focus of this scientific statement, it is instructive to ponder the role of hiPSC-CMs in the broader context of general personalized health care. Although most studies to date have relied on retrospective analysis of patient samples and medical histories, such studies must be cautiously interpreted and extrapolated because of potential limitations arising from selection bias. For an ideal predictive platform in the setting of personalized health care, a more rigorous approach requires prospective patient recruitment for hiPSC-CM studies to identify genetic, environmental, and other unique factors for testing cardiotoxicity susceptibility. However, such a prospective study would be years in duration and require hundreds of individuals to capture patients who will go on to experience cardiotoxicity. The success of such expensive endeavors would lead to preventive strategies for prophylactically mitigating cardiotoxicity with hiPSC-CMs. The cardio-oncological examples cited previously represent a first and necessary step in demonstrating the use of hiPSC-CMs in a rather specific application to personalized medicine that shows promise in the near future and will guide efforts toward more aspirational and broadly based prophylactic studies in more generalized personalized health care.

## CONCLUDING REMARKS: SHORT- AND LONG-TERM GAPS AND PATHS FORWARD

To be the most beneficial for patients, cardiotoxicity must be detected as early as possible, such as before the manifestation of clinical evidence of contractile dysfunction, especially in cases that lead to cascading or irreversible damage. Studies based on hiPSC-CMs are poised to lead the way for accurate assessment of direct cardiotoxic effects of oncological agents in the near future,[118] including the development of fit-for-purpose assays using various available preparations and approaches. Clinical studies evaluating the use of various biomarkers (eg, troponins, brain natriuretic peptide, microRNAs, and exosomes) for the early detection of cardiotoxicity are continuing and will provide guidance for nonclinical in vitro studies of cardiotoxicity using hiPSC-CMs. Furthermore, such studies are suggesting new clinical biomarkers to consider. There is an urgent need to coordinate such initiatives for all nonclinical studies using human-relevant preparations so that their strengths and limitations can be evaluated to guide their future use and development. Such collaborative efforts will reduce the potential for false-positive nonclinical cardiotoxicity findings for novel drugs that have plagued the development of lifesaving therapies. Further funding of academic, commercial, pharma, regulatory, and consortial initiatives is needed to continue these broad research efforts, with benefits to be accrued by all through safer drugs based on nonclinical evaluations using clinical-like cardiac preparations.

Continuing efforts to mature and refine hiPSC-CM–derived preparations to better recapitulate the complexity of mature ventricular myocardium will help us avoid unanticipated and unintended consequences of cardiotoxicity related to direct effects of evolving new pharmacological therapies, in part because, for the first time, hiPSC-CMs allow researchers to define and thereby avoid the direct mechanisms responsible for toxicity. Such studies with human-relevant preparations will move acute and shorter-term direct cardiotoxicity studies away from seemingly unpredictable and uncontrollable animal studies. Instead, more traditional animal studies will continue to serve as studies of cardiac injury resulting from more integrated responses involving additional components of the cardiovascular system and longer-term (or delayed) toxicities. However, neither approach addresses the potential direct effects of the destruction and removal of cancerous cells on the myocardium.

A looming challenge concerning the role of hiPSC-CMs in cardiotoxicity testing is to properly consider their utility for either hazard identification in early drug discovery or risk assessment in later drug development efforts. Such categorizations will ideally require defining types of reproducible fit-for-purpose preparations (ie, single cells, multicells, and 3D constructs) with differing functional and structural standards to compare with clinically recognized gold standards. The advantages of generating and using more complex human-relevant preparations, including greater maturity and more faithful recapitulation of native cardiac systems, need to be balanced against the reduced throughput, greater expense, and reproducibility challenges of these more complex preparations. The use of patient-specific hiPSC-CMs to screen for cardiotoxicity holds more immediate promise for adoption and integration into drug-induced cardiotoxicity safety testing. Of particular value is their use in

defining the safety of combination oncological therapies. However, much work still needs to be done to ensure reproducibility and to define acceptable standards of simpler versus more complex myocyte-based preparations and experimental protocols to avoid incorrect decisions guiding personalized patient care. It will be challenging to detect cardiac liabilities in early drug discovery to minimize patient risk and to reduce the use of animals while still ensuring the timely delivery of novel drugs and patient safety. The use of hiPSC-CMs has opened up multiple exciting opportunities to fulfill this role in human-relevant, mechanistic-based studies in the nonclinical arena.

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**Table 1.**

Human Relevant Myocyte Models for In Vitro Studies

| Construct | Composition and Description | Advantages | Disadvantages |
|---|---|---|---|
| Single hiPSC-CMs | May be micropatterned to form rectangles resembling adult myocytes | Amenable to electrophysiological studies using patch-clamp approaches | No intercalated disk or gap junctions<br>No syncytium function<br>Shape does not equal maturation<br>Single-cell isolation from sheets may cause injury |
| 2D layers | Cardiomyocytes grown in plates or wells in culture<br>Thin film/layers or sheet constructs possible | Ease of preparation<br>Amenable to HTS for electrophysiological, Ca flux, syncytial functions, measures of impulse propagation, fibrillation arrhythmias<br>Cells can be matured by media, patterning extracellular matrix manipulation | Lacks influence of other cardiac cell types and 3D environment of native tissue<br>Variable morphology and sarcomere alignment elicit strain patterns different from native myocytes |
| 2D cocultures | hiPSC-CMs mixed with fibroblasts, vascular cells, mesenchymal stem cells, native adult cardiomyocytes | Mimics heterogeneous cellular composition of native heart<br>Study cell-cell interactions, integrate hiPSC- CMs with noncontractile cells<br>Promotion of hiPSC-CM maturation | Optimum proportions of cell types uncertain<br>Proliferative cells may affect preparation stability and reproducibility<br>Nonmyocyte-to-myocyte coupling may differ from normal tissues |
| 3D organoids | hiPSCs cocultured with fibroblasts/ endothelial cells with self-assembly | Mimic 3D cardiac environment<br>Heterogeneous cell types may resemble native heart composition | Spatial arrangements of elements uncertain<br>Difficult to assess electrophysiology and contractility effects with multielectrode arrays and force measures |
| Engineered human tissues | hiPSC-CMs with/without fibroblasts, endothelial cells | Ability to directly measure contractile force, transmembrane potentials<br>Pacing controls rate<br>More natural alignment of cells/sarcomeres Enhanced myocyte maturation | Initial high myocyte requirements and costs being reduced by miniaturization<br>Lack of vasculature for thicker preparations may create diffusion barrier, anoxic core<br>Low to moderate throughput |
| 3D macroscopic constructs | Ventricular pouches Potential mini-ventricles | Direct pressure measurements possible May promote myocyte maturation | Technically demanding, high cell quantities needed<br>Limited to a few laboratories; low throughput; cost prohibitive |

A summary of different hiPSC-CM–derived models used to evaluate various cardiotoxicities. Models have been arranged in order of increasing complexity. In general, the protocols and composition of more complex structures increase the level of phenotypic maturity while challenging reproducibility and assay throughput.

2D indicates 2-dimensional; 3D, 3-dimensional; hiPSC, human induced pluripotent stem cell; hiPSC-CM, human induced pluripotent stem cell–derived cardiomyocyte; and HTS, high-throughput screening.

**Table 2.**

Approaches for Assessing In Vitro Cardiotoxicity With hiPSC-CMs

| Biomarker Category | Technological Approaches | Advantages | Disadvantages |
|---|---|---|---|
| Electrophysiology | Transmembrane recordings: action potentials (intracellular electrodes or VSDs)<br>Extracellular recordings: field potential measures (eg, multielectrode arrays); also conduction/propagation | Ability to assess drug effects on repolarization, depolarization, conduction, propagation; ion current measures with voltage clamp<br>MEA recordings enable longer-term studies of long-term effects and recovery (days to weeks)<br>VSD enables visualization of drug effects on action potential shape, providing insight into currents affected by drugs<br>Reflects biological integration of net effects on multiple ionic currents, exchangers, and pumps not fully reflected in in silico reconstructions | Variability of myocyte maturation may affect expression; key ion currents (Kir2.1, Nav1.5, $I_p$ etc) differ from native adult myocyte<br>Repolarization waveform from MEA recordings may be difficult to measure and interpret<br>Effects on repolarization using simpler surrogate measures (contractility, cell movement) may be challenging<br>Single-cell recordings possible but technically difficult and prone to potential artifacts from cell isolation |
| Contractility | Impedance measures linked to overall motion of myocytes sheets<br>$Ca^{2+}$ transient to assess EC coupling<br>Edge displacement and traction force to assess contraction and relaxation<br>Direct force measurements possible with thin film and myotube constructs | Measures represent surrogates of pump function with different translational fidelity | Disorganized sarcomeric structure, lack of t tubules in some constructs contribute to reduced force; calcium handling may be immature<br>Lack of anisotropic morphology may distort contractility assessments<br>Calcium-handling dysregulation linked to negative force-frequency relationship |
| Injury/structural damage | Measures of secreted/released proteins (troponins, proBNP), microRNAs, exosomes<br>Mitochondrial markers (mitochondrial membrane potential, morphology, number)<br>Morphology (cellular/organelle characteristics, cell viability markers)<br>Monitoring apoptosis/cytotoxicity with fluorescent dyes/time-lapse imaging | HTS is possible, full-dose responses, days to weeks of exposure possible; reversibility studies possible<br>Application of any substance to human heart tissue in vitro without risk to patients<br>Assessing early apoptosis induced by chemotherapy | Lack of neuronal and humoral influences present in intact myocardium<br>Difficult to replicate time course of in vivo exposures (parent and metabolite) in longer-term in vitro studies<br>Known resistance to hypoxic injury in some preparations<br>Immunological responses to removing products of cellular injury or dead cells are absent |

A summary of different approaches for assessing cardiotoxicity on the basis of toxicity type (electrophysiology, contractility, and injury/structural damage).

EC indicates excitation-contraction; hiPSC-CM, human induced pluripotent stem cell–derived cardiomyocyte; HTS, high-throughput screening; MEA, microelectrode array; proBNP, pro-B-type natriuretic peptide; and VSD, voltage-sensitive dye.