State of the heart : the promise of pluripotent stem cell-derived cardiomyocytes in disease modelling, differentiation and development

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CHAPTER 2

Pluripotent stem cell models of cardiac disease and their implication for drug discovery and development

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Abstract

Recent advances in pluripotent stem cell biology now make it possible to generate human cardiomyocytes in vitro from both healthy individuals and from patients with cardiac abnormalities. This offers unprecedented opportunities to study cardiac disease development 'in a dish' and establish novel platforms for drug discovery, either to prevent disease progression or to reverse it.

In this review paper, we discuss some of the genetic diseases that affect the heart and illustrate how these new paradigms could assist our understanding of cardiac pathogenesis and aid in drug discovery. In particular, we highlight the limitations of other commonly used model systems in predicting the consequences of drug exposure on the human heart.

Stem cells for cell therapies or pharmaceutical therapies from stem cells?

The use of stem cells to discover new pharmaceuticals rather than only as therapeutics is possibly among the most significant conceptual changes in stem cell biology of the past decade. In 2000, the perspective for human embryonic stem cells (hESCs) in clinical medicine centered almost entirely around their potential for cell replacement and tissue repair. Ten years on this remains a much sought ideal, but for many diseases and injuries it is clearly a distant goal. Instead, the ability to generate pluripotent stem cells (PSCs) from adult somatic cells, known as induced pluripotent stem cells (iPSCs) [1], and improved methods for directing differentiation, has shifted the focus to using PSCs for disease modeling and drug development. Cells of the heart are of particular interest because prolonged function of this organ is crucial to human health and yet can be jeopardized by malfunction of just one cell type – the cardiomyocyte.

The correct functioning of cardiomyocytes requires the coordinated interaction of multiple cardiac-specific proteins, most importantly ion channels and contractile proteins. A single mutation in one of these proteins can completely alter the response of the heart to physiological, pathological, drug-induced and electrical stimuli. Experimental animals, such as the mouse, guinea pig, rabbit and dog, cannot completely model human cardiomyocytes [2,3]. Because human PSCs can generate the major cardiomyocyte subtypes (atrial, ventricular and pacemaker cells) [4,5] and many cardiac diseases are autonomous to the cardiomyocyte, PSCs represent potential in vitro paradigms for understanding disease pathophysiology and for identifying pathways to target in ameliorating the conditions. Through knowledge of the molecular pathways involved in forming the heart and the identification of disease-causing genetic mutations, the stage has been set to exploit the new stem cell models for the benefit of human health. In this review paper, we introduce the cellular background to this research and discuss why some genetic cardiac diseases can be modeled in rodents, yet for others human cells are the only predictive option. We review recent PSC developments in the cardiac field, preview some of the new opportunities PSCs offer to drug discovery and comment on the challenges faced by the field.

The role of mice and cell models in understanding cardiac disease

Ion channels are pore-forming, transmembrane proteins that establish and control voltage gradients through the selective and directional flow of ions. The balance between depolarizing and repolarizing ion currents through these specialized channels creates the ‘action potential’ (AP) in electrically active cells such as cardiomyocytes [6] (Box 1). The AP essentially determines the electrical activity of the heart, although conduction and propagation through the

myocardium depends on the electrical coupling between cells, mediated by gap junctions. Mutations in genes encoding any of these proteins can cause abnormalities in ion channel function or cell-to-cell electrical coupling and can evoke life-threatening cardiac arrhythmias that can evolve into ventricular fibrillation, the most common cause of sudden cardiac death (Box 2). Conversion of electrical signals to mechanical responses is another important function of cardiomyocytes. Therefore, it is not surprising that cardiomyopathies, the other most common form of hereditary heart disease, are frequently caused by mutations in genes encoding the contractile sarcomere proteins [7].

Box 1. Electrophysiological differences between mouse and human

Humans exhibit significant differences in cardiac electrophysiology when compared with mice [6]. These differences are highlighted in the shapes and durations of their respective APs and ECGs (Figure I). Compared with humans, mice have a much shorter cardiac AP that lacks a clear plateau phase. This can also be easily visualized as the QT interval (a measure for the time between ventricular depolarization and repolarization) from an ECG that lasts between 50 and 100 ms in mice, whereas the human QT interval is approximately 400 ms.

The start of an AP is initiated by sodium channel activation and depolarization of the membrane potential (phase 0), followed by a brief repolarization (phase 1). This is subsequently followed by a prolonged inward flow of Ca^{2+}, which results in the plateau of the AP (phase 2). Following the inactivation of the Ca^{2+} channels, the delayed and inward rectifier K^+ channels are activated, resulting in the repolarization of the cell (phase 3) and the end of the AP (phase 4). Although the rapid upstroke of the AP, caused by the influx of sodium (Na^+) ions through the voltage-gated Na^+ channel, is similar in both mouse and human, there are major differences in the repolarization phase of the AP between the two species. The human ventricular AP has two main repolarizing currents, the rapid and the slow components of the delayed rectifier potassium (K^+) currents, I_{Kr} and I_{Ks}. By contrast, the main current responsible for mouse AP ventricular repolarization is the transient outward K^+ current, I_{to}, whereas in humans this exclusively contributes to phase 1 of the repolarization. Furthermore, in mouse but not in human ventricular myocytes, the delayed rectifier K^+ currents, $I_{K,slow1}$ and $I_{K,slow2}$, and a steady state K^+ current (I_{ss}) are responsible for AP repolarization. A functional consequence of these differences in voltage gated K^+ channel expression is that genetic mouse models of human LQT1 and LQT2, which are associated with loss of I_{Ks} and I_{Kr} currents, respectively, do not fully reproduce the human phenotype.

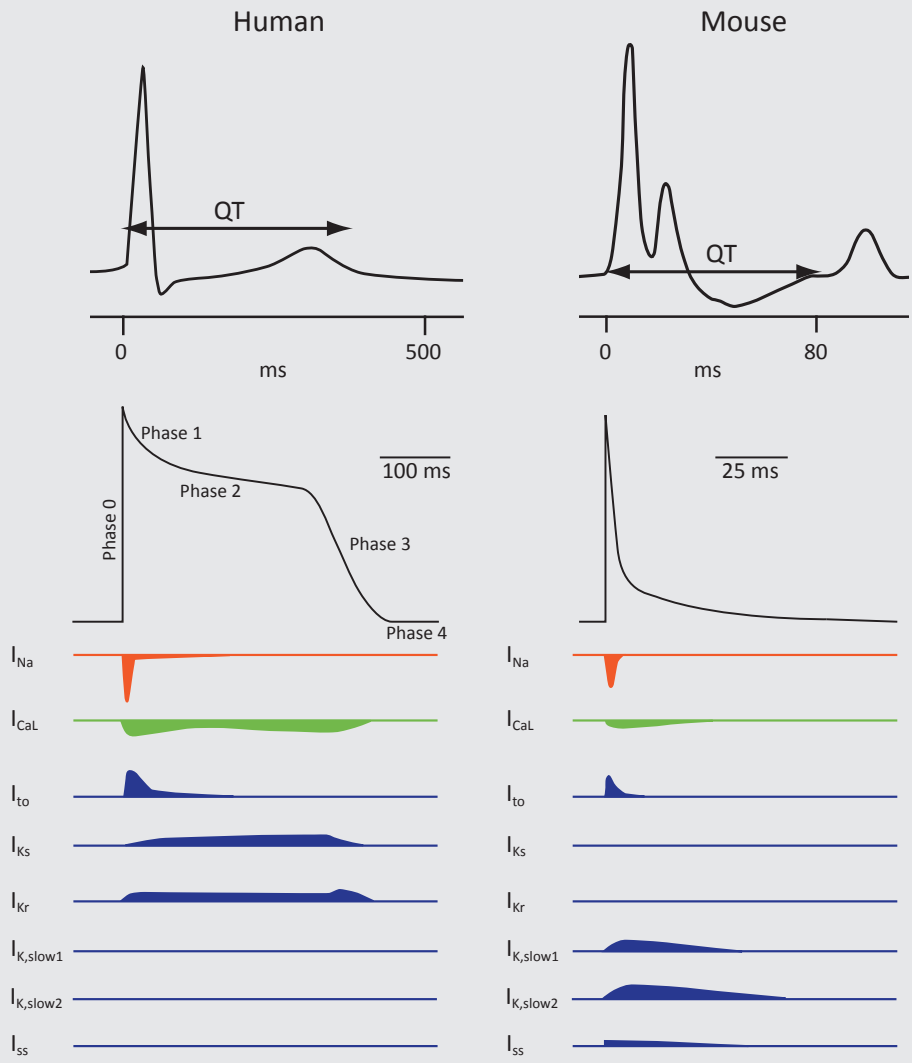

Figure I. Electrophysiological differences between adult human and mouse CMs
Human and mouse hearts display unique ECG profiles, with the QT interval in humans approximately five times longer than in mice. This is also reflected in the different AP durations and shapes of the ventricular CMs from these two species and is due to their distinctive ionic currents. Although the inward Na^+ and Ca^{2+} currents (I_{Na} and I_{CaL}, respectively) are comparable between mouse and human CMs, major differences are observed in the various outward K^+ currents (in blue), which play distinct roles in human and mouse AP repolarization. The I_{Ks} and I_{Kr} are the major repolarizing currents in human CMs, whereas in mice I_{to} is the predominant current. Additionally $I_{K,slow1}$, $I_{K,slow2}$ and I_{ss} contribute to repolarization in mouse CMs, but are absent from human ventricular CMs. The APs and their underlying ionic currents are aligned with their approximate time of action during the QT interval shown in the ECG examples. ms, millisecond.

Box 2. The cardiomyocyte and its maladies

Syndromes resulting from ion channel mutations are termed congenital cardiac channelopathies and, depending on the gene affected, include Brugada disease, LQT, short QT (SQT) and catecholaminergic polymorphic ventricular tachyarrhythmia (CPVT) [60]. They are often caused by single genetic mutations that result in distinctive abnormalities evident on an ECG. LQT, the most common EP disorder has 12 different subtypes. It is characterized by prolongation of the AP duration (APD) and results in an extended QT interval on an ECG. Mutations in the genes encoding potassium *(KCNQ1*, *KCNH2*), sodium (*SCN5A*) and calcium (*CACNA1C*) channels are the most common cause of the syndrome, which is usually inherited in an autosomal dominant manner.

The two most common forms of inherited myopathies are hypertrophic (HCM) and dilated (DCM) cardiomyopathy. Both can ultimately lead to reduced pumping efficiency of the heart and congestive heart failure (HF). The majority of the mutations have been found in two genes, myosin heavy chain 7 cardiac muscle β (*MYH7*) and myosin binding protein C (*MYBPC3*) [7]. Like EP disorders, cardiomyopathies are mostly inherited as autosomal dominant traits.

Genetic mouse models of human LQT1 and LQT2 do not fully reproduce the human disease phenotype (Box 1). Another example of the species difference between mice and human is illustrated by phospholamban (PLN), a regulator of cardiac Ca^{2+} homeostasis that is involved in myocardial relaxation and cardiac contractility. PLN has been long considered an interesting therapeutic target in diseases such as cardiomyopathy and heart failure, where Ca^{2+} handling is disturbed. This is partly because PLN-deficient mice exhibit enhanced Ca^{2+} re-uptake and improved contractility [61]. Furthermore, crossing these mice into two mouse cardiomyopathy models rescued the disease phenotype [62,63]. However, the excitement of these results was tempered by the finding that PLN ablation failed to rescue the cardiac dysfunction in two other mouse models of genetic HCM [64]. Additionally, humans lacking PLN due to a genetic mutation show no signs of improved contractility, instead developing severe dilated cardiomyopathy and HF [65]. PLN ablation is therefore unlikely to be a universal approach for treating all forms of HF. However, further studies using hiPSC-CMs from both healthy individuals and from HF/HCM patients could clarify under what genetic and environmental conditions lowering PLN levels might be beneficial in treating these diseases.

Much of our current knowledge on the signaling pathways causing cardiac diseases has come from heterologous expression systems and genetic animal models [8-12]. Mice have made a major contribution because they can be easily genetically modified, maintained and bred. However, considerable differences exist between the human and mouse genomes, with many genetic modulators being human-specific. Additionally, mice and humans differ in cardiac physiology. For example, the resting heart rate differs tenfold and physiological responses to exercise and arrhythmias are distinct [6].

Cell culture has been almost as crucial to research on cardiopathies as animal models. Because primary cardiomyocytes are difficult to isolate and have a short lifespan in culture, genetically transformed cell lines or immortalized cells from tumors are usually utilized. However, these surrogate cell lines are often genetically abnormal and can carry other genetic and epigenetic artifacts from long-term cell culture [13]. Also, mutant ion channels artificially overexpressed in these heterologous cell systems lack the correct cellular context of a cardiomyocyte. For example, hERG channels expressed ectopically in HEK293 cells behaved differently from those found in neonatal rat cardiomyocytes [14]. Similarly, an ankyrin-binding site mutation in the Scn5a ion channel prevented its cell surface accumulation in rat ventricular cardiomyocytes, although no trafficking problem was observed in non-cardiac cells [15].

PSCs offer an unlimited source of cardiomyocytes

Because of the shortcomings in the model systems described above for investigating cardiac function and pathogenesis, alternative models based on cardiomyocytes derived from human PSCs (namely hESCs and hiPSCs) are of interest. Undifferentiated PSCs can proliferate for extended periods in culture but can also differentiate into various cell types, including rhythmically contracting heart muscle cells [4,5]. These PSC-derived cardiomyocytes (PSC-CMs) express appropriate ion channel, signaling and contractile proteins for functional excitation-contraction coupling [5,16].

Since the first derivation of hESCs from normal embryos in 1998 [17], the possibility of using these cells to model human genetic diseases has existed. However, initial difficulties in genetically modifying hESCs and the ethical and legislative issues associated with destroying surplus embryos during derivation restricted research in many countries and limited their use among the wider research community. The discovery that adult somatic cells could be reprogrammed to a pluripotent state by the overexpression of four transcription factors (*Sox2*, *Oct4*, *c-Myc* and *Klf4*) [18] was a breakthrough in stem cell biology that has resulted in many new groups entering the field. Although hiPSCs might not be epigenetically identical to hESCs [19], they clearly self-renew and are pluripotent. Since the first report describing iPSCs, rapid progress has been made in reprogramming various somatic cell types using many variants on the original cocktail of reprogramming genes [20], including replacing some with chromatin-modifying small molecules [21] (Figure 1). Similarly, numerous methods for delivering the reprogramming factors to the cell, including non-integrating genomic approaches, have been published [20,22].

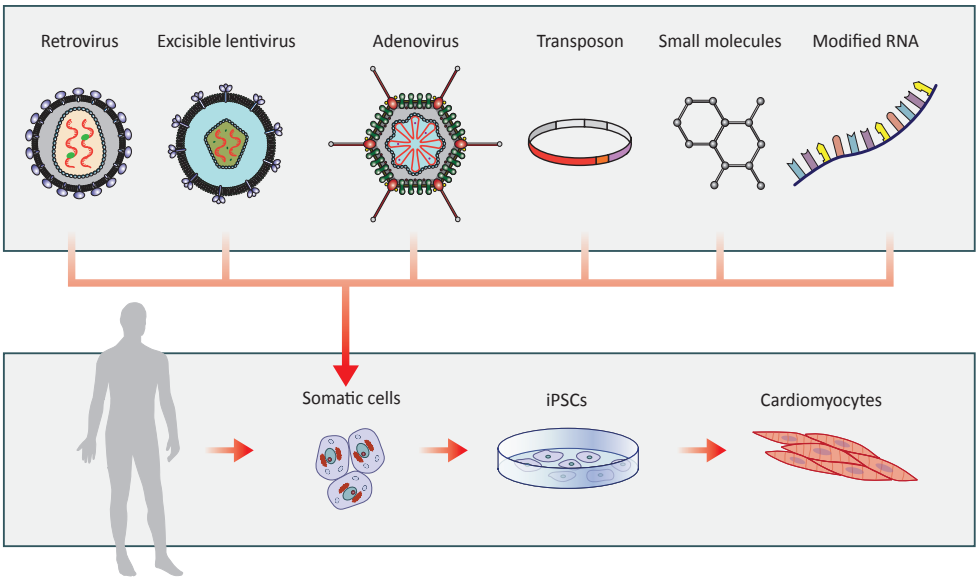

Figure 1. Generation of hiPSCs
In humans, iPSC lines have been generated from a range of somatic cell types, such as fibroblasts, keratinocytes, adipocytes and terminally differentiated lymphocytes. These somatic cells can be reprogrammed to hiPSCs by the overexpression of the pluripotency-associated genes, *OCT4*, *SOX2*, *C-MYC* and *KLF4*. Some of these transcription factors can be replaced by other genes or, depending on the somatic cell type, excluded. The reprogramming genes can be delivered into the somatic cells by a variety of approaches including retroviruses, lentiviruses or adenoviruses, or by the use of transposons. The continued presence of the reprogramming factors in the iPSCs can have adverse effects on their cellular function, and therefore generating integration-free iPSCs efficiently is desirable. This can be done by using vectors that do not integrate into the host cell genome (adenoviruses) or by using integrating vectors that can be subsequently removed from the genome (excisable lentiviruses or transposons). Recently, integration-free hiPSCs have been produced by using modified RNA molecules to introduce the reprogramming factors into the somatic cells. Additionally, several small molecules, such as the histone deacetylase inhibitor valproic acid (VPA) or the transforming growth factor β (TGF-β) signaling inhibitor E-616452, can either increase the reprogramming efficiency or replace individual reprogramming factors. The resulting hiPSC lines can then be differentiated in vitro into cardiomyocytes (Box 3).

Aside from circumventing the ethical issues associated with hESCs, hiPSCs have made it possible to generate PSCs and their derivatives from any individual of choice and, in principle, provide an autologous source of cells for future therapy. However, this level of personalized medicine is unlikely to become a reality in the short term, not least because of the time and cost required for deriving hiPSC lines and differentiating them to the cell type necessary without residual undifferentiated cells. Rather, these cells are more likely to be utilized to model genetic diseases in vitro. This could be particularly advantageous for studying diseases that result from chromosomal translocations or deletions, or when the genetic variant that causes the disease is unknown, as this would currently be very difficult to model in

hESCs. However, it is crucial to consider the most appropriate control hiPSC line with which to compare the patient-derived lines because differences in genetic background between the lines could confound distinguishing the disease phenotype from natural variations in phenotype.

Alternatively, PSC models for monogenetic diseases could be developed using hESCs derived from diseased embryos or by introducing the variant sequence into the gene. Although homologous recombination in hESCs initially proved challenging, adaptations to culture conditions improved efficiencies [23,24], and several targeted hESC lines have been reported (summarized in [25]). Compared to hiPSC-based models, this approach offers the benefit that the parental hESC line serves as the control with the only genetic difference between the lines being the introduced mutation. Alternatively, the genetic variant could be corrected in the patient-derived hiPSCs.

Essential for modeling cardiac disease are robust, reproducible differentiation protocols to obtain functional cardiomyocytes in vitro (Box 3). The most successful approaches to date expose PSCs to similar developmental cues that occur in early embryonic development [26]. These include signaling by bone morphogenetic proteins (BMPs), Nodal/Activin A and fibroblast growth factors to induce mesoderm formation and specification, which precedes cardiogenesis. Signaling through the canonical Wnt pathway also plays discrete time-specific roles, with expression of Wnts promoting the formation of cardiogenic mesoderm, but repressing the subsequent specification to cardiomyocytes [27]. Functional cardiomyocytes have been derived in vitro from both hESCs and hiPSCs [4,5], although they are typically immature with irregular sarcomeres and membrane potentials, with upstroke velocities and AP amplitudes comparable to 16-week-old fetal hearts [4]. Therefore, disease pathologies might not be evident if the disease-causing mutation occurs in a gene that is not expressed until later in development or if the disease occurs postnatally.

PSC lines modeling cardiac disease

Although no hESC lines modeling a cardiac disease have been published to date, there are now several reports of hiPSC lines from individuals with gene mutations affecting the heart [28-32]. Although there was evidence for the expected cardiac disease phenotype in all cases, the controls for comparison were cardiomyocytes from an unrelated line and none of the studies corrected the mutation to confirm that it was the pathogenic variant. Over the next few years, the number of cardiac disease lines available from both hESCs and hiPSCs will undoubtedly increase, with ion channelopathies and cardiomyopathies lacking suitable animal models being of particular interest. It is probable that these models will be useful for studying disease processes and evaluating novel therapeutic strategies (Box 4). Below, we discuss some of the information these studies have provided us with so far.

Box 3. Methods to differentiate human PSCs to cardiomyocytes

One of the major challenges in PSC research is to develop reproducible and robust differentiation strategies to derive desired cell types in sufficient quantities for downstream analysis. In the present context, this means first to cardiogenic mesoderm, then to cardiac progenitors and finally to functional cardiomyocytes. Presently, there are three predominant strategies to differentiate both hESCs and hiPSCs to cardiomyocytes.

Coculture with END-2 stromal cells

This in vitro differentiation method utilizes signaling molecules secreted by mouse visceral endoderm-like (END-2) cells, to induce PSCs to form cardiomyocytes [4]. Although the overall yield of cardiomyocytes is low, the method has been used to successfully differentiate more than 10 hESC and hiPSC lines to cardiac cells, demonstrating the general applicability for testing the cardiogenic potential of newly derived PSCs.

Embryoid body (EB) differentiation

This technique involves culturing ESCs in suspension in the presence of differentiation signals (e.g. TGF-β factors, Wnts), resulting in the cells differentiating and forming three-dimensional aggregates commonly referred to as embryoid bodies (EBs). Many protocols involve harvesting the undifferentiated PSCs by either manual dissociation or collagenase [66]. Although spontaneously beating areas within the EB can be detected as early as day 7 of differentiation, there is also often heterogeneity in the EB size and limited cardiomyocyte differentiation, partially owing to the differences in cell numbers that seed each EB. A variant on this method involves the forced aggregation by centrifugation of PSCs dissociated to single cells [67,68]. This makes it possible to form EBs from defined numbers of cells, improving the reproducibility within and between experiments. Additionally, this method is amenable to higher throughput applications because the procedure is carried out on 96- or 384-well plates. With the 'Spin EB' method, differentiation can be performed in a chemically defined, serum-free medium [69], enabling the cardiomyogenic inductive effects of growth factors and small molecules to be systematically analyzed.

Monolayer differentiation

One of the ultimate aims is to be able to differentiate PSCs under fully defined conditions without forming EBs and derive specific mature cell types in large quantities. It has been demonstrated that cardiomyocytes can be obtained from monolayer cultures in which the hESCs were seeded at high density on matrigel [70]. However, it remains unclear whether this method is translatable to other PSC lines.

Box 4. Identifying abnormalities in PSC-derived cardiomyocytes

Once cardiomyocytes have been derived from control or disease-bearing human PSCs, it is important that any phenotypic differences can be measured both qualitatively and quantitatively, in addition to any drug-induced response. These readouts can be based on the characteristic EP, morphological and contractile properties of the PSC-CMs (Figure II).

For models of EP disorders, the most facile observation is modulations to the contractile frequency of the PSC-CMs in response to pharmacological agents that induce arrhythmias in corresponding groups of patients. However, more in-depth characterization of the electrical signaling in the cardiomyocyte is also required. This can be performed by patch clamp analysis to assess the intracellular AP, as well as to study the activity of the ion channel affected by the genetic mutation. Alternatively, a microelectrode array (MEA) can measure the extracellular field potentials of clumps of cells, which can be correlated to APD and QT interval. Optical mapping techniques to measure the spread of electrical activation and repolarization among excitable cells, in combination with recent tissue engineering advances to generate two-dimensional models of heart muscle, will also be valuable tools for measuring differences between healthy or diseased cell lines. Most mapping studies use a fluorescent dye that is either voltage- or calcium-sensitive, with changes in fluorescence indicating the activity of particular ion channels and transporters.

Cell imaging and immunohistochemistry are also important tools for analyzing the hypertrophic response of PSC-CMs modeling HCM. Compared with wild-type PSC-CMs, hypertrophic PSC-CMs typically have larger cell surface areas and volumes and can display sarcomeric disorganization. Although multiple parallel signaling pathways are altered during pathological hypertrophy, common key players have been identified. For instance, activation of the phosphatase calcineurin leads to translocation of the transcription factor NFAT from the cytoplasm to the nucleus to activate a hypertrophic genetic program [71]. These observations can be used as basic measures for demonstrating hypertrophy. Additional assays that can be performed include determining the total cellular protein to DNA content ratio, with hypertrophic CMs expected to show an increase, and gene expression analysis for hypertrophy-related structure proteins, transcription factors, calcium modulators and atrial natriuretic factor (ANF). Finally, it has been proposed that HCM mutations can lead to inefficient ATP-utilization of the sarcomeres, increasing energy demand and altered Ca^{2+} handling, which results in contractile dysfunction [72]. Reduction in the phosphocreatine/ATP levels and impaired contractility detected using Ca^{2+}-sensitive dyes could also be used as readouts.

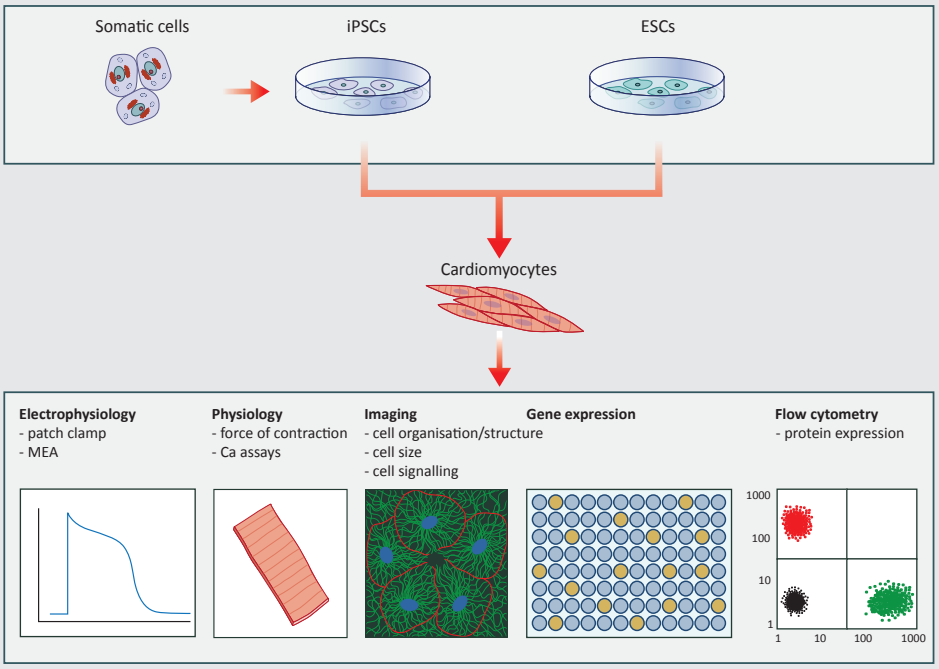

Figure II. In vitro properties that can be quantitatively measured and compared between cardiomyocytes derived from PSC disease models
ESC or iPSC lines containing a genetic mutation associated with cardiac disease, along with the appropriate control cells, can be differentiated into cardiac cell types. Assays based on electrophysiology, physiology, morphology, or gene or protein expression can be performed on these cells, enabling the underlying mechanism of the disease to be investigated, as well as any potential therapeutic interventions to be assessed.

Ion channelopathies

To date, hiPSC lines derived from patients afflicted with various long QT (LQT) syndromes (LQT1, LQT2 and LQT8) have been reported. Patients with LQT syndromes show characteristic extensions of the QT interval on an electrocardiogram. For LQT1, hiPSC lines were derived from a father and son carrying an Arg190Gln missense mutation in *KCNQ1*, the gene encoding the pore-forming subunit of the potassium channel generating I_{Ks} [29]. Although mouse models with mutations in *Kcnq1* are available [6], there are significant differences in the handling of potassium currents between mouse and human (Box 1). The report demonstrated that the prolonged QTc interval (QT interval corrected for heart rate) observed in electrocardiograms (ECGs) from the two patients was recapitulated in the hiPSC-CMs, with ventricular APs significantly longer than in control hiPSC-CMs. Further electrophysiological analysis demonstrated that I_{Ks} density was decreased and channel activation and deactivation altered in the patient-derived iPSC-CMs. Of particular note was that adrenergic stimulation

(a known arrhythmic trigger in LQT1 sufferers) exacerbated the number of arrhythmic-like events and that β-blockade attenuated the phenotype, illustrating the potential use of hiPSC-CMs from specific individuals in investigating hypersensitivity to particular drugs. Additional characterization of the Arg190Gln–KCNQ1 mutation in the hiPSC-CMs indicated that the reduction in I_{Ks} was most probably due to a trafficking defect. However, further studies to confirm this were performed in the rat cardiomyoblast cell line, H9c2, highlighting the current difficulties in isolating sufficient cardiomyocytes from human PSCs for such investigations.

Two other studies examined the role of missense mutations in *KCNH2* from sufferers of LQT2 [30,31]. This gene encodes the pore-forming subunit of the hERG potassium channel responsible for I_{Kr}. The importance of this channel for normal electrical activity is highlighted by the withdrawal of several medications found to block the hERG channel, triggering ventricular arrhythmias and sudden death [33]. Prolongation of the AP duration (APD) was observed in the Ala614Val–KCNH2 and Ala561Thr–KCNH2 hiPSC-CMs compared with control hiPSC-CMs, owing to a reduction in I_{Kr}. Although the resulting phenotype is similar for both mutations, the underlying molecular mechanisms are believed to be different based on prior heterologous expression studies [34,35]. It will be interesting to determine whether these hiPSC lines confirm these previous findings. Additionally, both studies observed early-after depolarizations (EADs) in some of the patient iPSC-CMs. These spontaneous membrane depolarizations can trigger the onset of ventricular arrhythmias [36]. Notably, while APD increases were observed in hiPSC-CMs derived from an asymptomatic carrier of Ala561Thr–KCNH2, β-adrenoreceptor stimulation did not lead to EADs [31]. This was consistent with clinical data for this patient, raising the possibility that hiPSCs might be able to model variations in phenotype between different patients carrying the same mutation and assist in identifying additional polymorphisms in the genome that might be responsible.

Finally, another group derived hiPSC lines from two patients suffering from Timothy syndrome (LQT8), caused by mutation in the gene encoding the L-type calcium channel $Ca_V1.2$ (*CACNA1C*) [32]. The Gly406Arg variant is a gain-of-function mutation that severely impairs voltage-dependent inactivation and subtly affects Ca^{2+}-dependent inactivation of the channel [37]. In contrast to the other LQT hiPSC models, only ventricular-like myocytes derived from the Gly406Arg–$Ca_V1.2$ hiPSCs had prolonged APs, as well as excess Ca^{2+} influx, irregular electrical activity and abnormal calcium transients. The link between Timothy syndrome and mutations in *CACNA1C* was only recently discovered, thus the mechanism underlying how the Gly406Arg variant precisely leads to LQT or arrhythmias in humans is unclear. It is probable that these new hiPSC models will assist in these studies.

Cardiomyopathies

Although hypertrophic cardiomyopathy is often due to a mutation in one of the sarcomeric genes, it can also develop owing to storage diseases or other metabolic abnormalities [38,39]. Patients with LEOPARD syndrome can suffer from various symptoms, with hypertrophic cardiomyopathy being the cause of mortality in some patients [40]. This autosomal-dominant hereditary disorder is commonly caused by mutations in the ubiquitously expressed tyrosine phosphatase gene, *PTPN11*. hiPSCs, generated from two patients with one of the most recurrent mutations, Thr468Met, were differentiated to cardiomyocytes and assessed for hypertrophy [28]. The hiPSC-CMs displayed greater sarcomere organization and nuclear NFATC4 localization, and were larger than hESC-CMs or wild-type hiPSC-CMs. Although only a few of the standard cardiomyocyte hypertrophy criteria were analyzed owing to the limited availability of cardiomyocytes, recently improved differentiation and enrichment strategies for cardiomyocytes [41-43] augur well for modeling this disease phenotype with PSCs.

PSC disease models in drug discovery and development

With cardiac disease being the primary cause of morbidity and mortality in aging populations of the Western world [44], and with a paucity in therapeutic interventions, the identification of novel and experimental cardiac drug targets has recently received renewed interest. As discussed earlier, one reason for slow cardiac drug target discovery (and development) is the limited predictive value and high costs of animal-based model systems. Over the past few years, the multiplicity of new start-up companies in the iPSC field and active interest from pharmaceutical companies illustrate the expected contribution of human PSCs to various phases of drug discovery and development.

Classical drug target discovery relies on a thorough understanding of human biology, with comparisons of the healthy and diseased state used to identify potential drug targets (Figure 2, top) [45]. However, despite years of genetic, biochemical and cellular research, the mechanisms underlying many cardiac disorders remain unknown. Genome-wide association studies (GWAS) have linked (noncoding) DNA variants with specific cardiac diseases [46], but it has been difficult to confirm the impact of these gene variants through experimental functional assessment in disease models. Many candidate loci also have no clear ortholog in mice or other non-human species. Because hiPSCs could be derived from some of the patients used in the initial GWAS, it might be possible to determine which loci or pathways are responsible for, or are associated with, predisposition towards a cardiac disease.

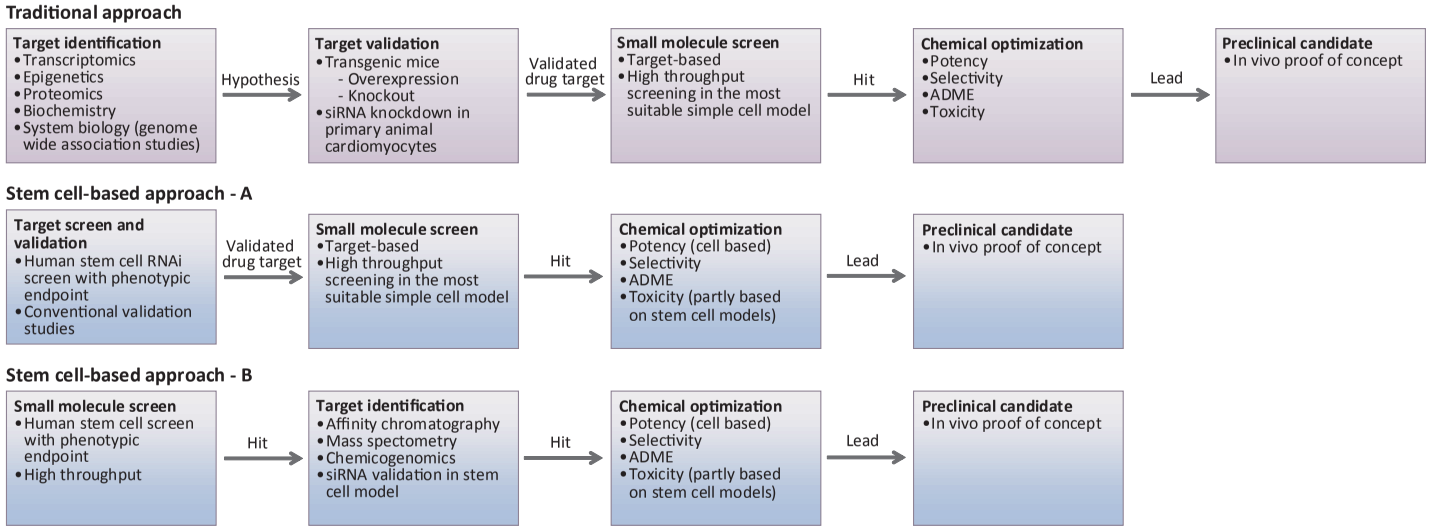

Figure 2. Current and future cardiac drug discovery approaches (modified from [59])

Traditional approaches to drug target discovery involve performing basic research on disease mechanisms first, usually using standard genetic and biochemical techniques, and this information identifies new potential drug targets. These targets are validated in cultured cardiomyocytes (if available) and transgenic or knockout mice, a process often taking many years with several possible points of failure. Consequently, the most important step, small molecule screening for agonists or antagonists of the targets, can be delayed. An alternative approach could be 'phenotype-based' high-throughput screens using hiPSC-CMs in combination with (**A**) siRNA libraries or (**B**) chemical libraries to identify novel drug targets and small molecules, respectively. These unbiased approaches on *bona fide* human cardiomyocytes could identify drug targets or classes of compounds that elicit a desired effect without a deep understanding of the biological processes *a priori*. At later stages, optimized lead compounds could be tested in the same cell model to understand the underlying mechanisms, thereby accelerating translation of preclinical discoveries to clinical testing. siRNA, small interfering RNA; ADME, absorption, distribution, metabolism and excretion.

Cell-based screening for drug discovery

Although PSC technology could be instrumental in understanding the biology of healthy and diseased cardiomyocytes, this might not be enough for efficient drug target identification. Over the past two decades, several gene mutations causing cardiac disease have been identified through candidate gene approaches in families exhibiting hereditary cardiac abnormalities, but it has proven extremely difficult to translate this to the identification of drug targets and therapies.

Cell-based screening with phenotypic endpoints is now proving successful in some disease areas [47]. Typically, primary human cells are used to create disease models in culture with a finite 'window for screening'. These models can be used in combination with either RNAi to find drug targets [48,49] or chemical libraries to find novel lead molecules [50] (Figure 2, middle and bottom). For reasons mentioned earlier, primary human cardiomyocytes are unsuitable for high-throughput screening, but human PSC-CMs might solve this problem. Human PSC disease models that accurately reflect the disease pathology could be developed into miniaturized high-throughput and high-content phenotypic screens (HTS/HCS). Although specific HTS/HCS assays for PSC-CMs have not been described yet, a range of assays to identify cell responses have been described for other cell sources [51]. Using PSC-CMs, one might be able to design a complex assay based on simultaneous imaging and measurement of parameters such as cell proliferation, differentiation, morphology and viability. Recent reports have provided promising preliminary data that hiPSC-CMs from disease models display expected responses to anti-arrhythmic compounds and can also be used to validate the efficacy of experimental therapeutic interventions [30,31].

Personalized medicine and stem cell banks

The late-stage attrition of drugs due to limited effectiveness or unforeseen side effects is perhaps the biggest problem in drug development. This is in part because not all individuals receiving a particular drug will benefit or they will suffer toxic side effects. This has resulted in tighter legislation and increased costs to bring a drug to market. Therefore, there is a growing interest in developing more personalized, predictive and preventive medications [51]. It is widely acknowledged that biobanks containing well-documented patient material could help achieve this goal. Cryopreserved somatic cells could be an extremely valuable addition to current biobanks because they could be potentially used to generate iPSCs. A panel of PSC lines with different genotypes might allow in vitro analyses predicting both drug effectiveness and toxicity at an individual level against a specific genetic background, as well as identifying biomarkers for the development of companion diagnostic assays to predict individual therapy response and toxicity in the clinic. This assumes that genotypic variation is retained during reprogramming, which remains to be proven.

Future challenges and considerations

Although many laboratories are developing cardiovascular disease models using human PSCs and the overall disease phenotype of the patient is apparently retained in these cells, there are several technical issues to be addressed before these PSC-CMs can be successfully implemented in the drug discovery and development pipeline. One of the key criteria is the availability of efficient and robust differentiation protocols that are applicable to a variety of PSC lines. Recently, small changes in the levels and timing of Activin/Nodal and BMP signaling when differentiating PSCs was shown to dramatically alter the proportion of cardiac mesoderm generated [43]. Although optimization of conditions was required for each individual PSC line, it provides a clear starting point and framework to develop improved differentiation protocols.

The maturity of the PSC-CMs derived also needs to be considered. PSC-CMs typically have an immature phenotype, with a gene expression profile resembling that of fetal cardiomyocytes [52]. Additionally, they present less negative resting membrane potentials, lower upstroke velocities and smaller AP amplitudes compared with adult cardiomyocytes, and their sarcomeres and shapes are relatively disorganized and not aligned as in adult cells [4]. Although for many applications an immature PSC-CM model might still offer an improvement over traditional model systems, it remains a challenge to understand and recapitulate in vitro the physiological and biochemical triggers active in normal development.

Once the desired types of cardiomyocytes can be produced in sufficient quantities, it will be essential to develop predictive assays at an appropriate level of throughput. For example, an arrayed RNAi-based target discovery library screen typically involves three siRNAs directed against the whole druggable genome (~5000 genes). At the other extreme, testing of efficacy in lead optimization might be restricted to a few candidate compounds. During assay development, it should also be remembered that the heart is not only composed of cardiomyocytes (~30 % of the total cells present) but also two other principal cell types, cardiac fibroblasts (~60 %) and vascular endothelial cells (~10 %). Although most electrophysiological (EP) disorders and cardiomyopathies directly affect cardiomyocytes, for other cardiac diseases these other cell types might also influence cardiomyocyte function. Additionally, many electrophysiology assays rely on isolated single cardiomyocytes. Maintaining the myocytes in this format might alter their cellular function and does not take into account the intercellular electrical coupling of cardiomyocytes in the heart. Therefore, a miniaturized HTS/HCS-compatible stem cell-based model that recapitulates cardiac organogenesis, essentially creating an artificial human myocardium, could be invaluable.

Although this review has focused on inherited forms of cardiac disease, the majority of cardiac conditions are acquired, often as a result of abnormal physiological insults. In the intact human heart, these can include lack of oxygen during a myocardial infarction, high

blood pressure, high glucose levels due to diabetes and changes to cardiomyocyte shape during heart failure or cardiac remodeling following post-myocardial infarction [53]. In some cases, these changes can be recapitulated in the laboratory using chemicals or drugs such as phenylephrine [54], or in the case of shape changes, by micropatterning extracellular matrix proteins such as fibronectin onto synthetic polymers [55]. Development of new methods to induce pathological cardiac stress in culture is eagerly awaited. Alternatively, monogenetic disease PSC lines might be adequate surrogate models to gain insights into the biology of some acquired disorders such as acquired LQT.

More general questions on iPSCs might also require further clarification before their true potential to model cardiovascular diseases is known. Recent studies have shown that the reprogramming process and subsequent culture of iPSCs in vitro can induce genetic and epigenetic abnormalities [56]. Although these lesions could limit the use of hiPSCs in therapeutic applications, they might not be of such concern for in vitro studies provided the pathogenesis of the cardiac disease is unaffected by these changes. Similarly, iPSCs have been reported to retain an epigenetic memory of their somatic cell source with a tendency to differentiate more efficiently to lineages of the donor cell [57]. However, this residual memory does not appear permanent, as continuous cell division appears to equilibrate the differentiation capacity of iPSCs derived from different tissue sources [58].

Also, how closely will the in vitro disease phenotype in the iPSC models match that of the patient from whom they were derived? In some familial hypertrophic cardiomyopathies, examples are known where the cardiac septum is ten times thicker than normal in one family member, yet another family member with the same mutation has no apparent symptoms. It remains to be seen whether such obvious differences in patient phenotypes are genetically based, and whether similar differences will be reflected in the cardiomyocytes derived from their respective iPSC lines. Answers to the question of individual genetic background effects might, in some cases, be found in mice by breeding identical mutations into different congenic backgrounds and determining whether the phenotype in the animals is phenocopied in the mouse iPSCs.

Finally, it is worth considering what we can learn about the disease from PSCs that cannot be gleaned from studying the patients. For example, in many electrophysiological disorders, the physiological basis for the arrhythmia is known. It remains to be seen whether human PSC models for genetic ion channelopathies can help us further understand the molecular mechanism underlying the arrhythmic trigger and lead to the development of new therapeutic interventions. Recent reports provide optimism that this will be possible [29-32], although a wider range of cardiopathy models from both iPSCs and ESCs are still required to determine the reliability of observing the in vivo phenotype in vitro.

Concluding remarks

Over the past several years, a number of major technical advances in PSC biology have created opportunities that will have a significant impact on our understanding of human diseases. Aside from applications in cell-based therapies, we have discussed here the potential use of human PSCs to model normal development as well as aspects of disease. Congenital cardiac diseases are at the forefront of these applications, in part because these diseases are autonomous to cardiomyocytes, and can therefore be investigated using in vitro cardiomyocyte cell models. Protocols for deriving these cells in culture are also continually improving, with more reproducible differentiation strategies that produce cardiomyocytes in numbers amenable for applications such as drug discovery and biomarker screening becoming available. It has already been demonstrated that arrhythmic phenotypes can be observed in hiPSC-CMs derived from LQT patients, a concept inconceivable just five years ago. The challenge now is to use PSC disease models to gain new insights into the molecular mechanisms leading to the disease and to use this knowledge to generate new therapeutic interventions.

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