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Induced Pluripotent Stem Cells for Cardiovascular Disease Modeling and Precision Medicine:

A Scientific Statement From the American Heart Association

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Abstract

Induced pluripotent stem cells (iPSCs) offer an unprecedented opportunity to study human physiology and disease at the cellular level. They also have the potential to be leveraged in the practice of precision medicine, for example, personalized drug testing. This statement comprehensively describes the provenance of iPSC lines, their use for cardiovascular disease modeling, their use for precision medicine, and strategies through which to promote their wider use for biomedical applications. Human iPSCs exhibit properties that render them uniquely qualified as model systems for studying human diseases: they are of human origin, which means they carry human genomes; they are pluripotent, which means that in principle, they can be differentiated into any of the human body's somatic cell types; and they are stem cells, which means they can be expanded from a single cell into millions or even billions of cell progeny. iPSCs offer the opportunity to study cells that are genetically matched to individual patients, and genome-editing tools allow introduction or correction of genetic variants. Initial progress has been made in using iPSCs to better understand cardiomyopathies, rhythm disorders, valvular and vascular disorders, and metabolic risk factors for ischemic heart disease. This promising work is

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still in its infancy. Similarly, iPSCs are only just starting to be used to identify the optimal medications to be used in patients from whom the cells were derived. This statement is intended to (1) summarize the state of the science with respect to the use of iPSCs for modeling of cardiovascular traits and disorders and for therapeutic screening; (2) identify opportunities and challenges in the use of iPSCs for disease modeling and precision medicine; and (3) outline strategies that will facilitate the use of iPSCs for biomedical applications. This statement is not intended to address the use of stem cells as regenerative therapy, such as transplantation into the body to treat ischemic heart disease or heart failure.

Keywords

AHA Scientific Statements; models, cardiovascular; precision medicine; stem cells, induced pluripotent

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Human pluripotent stem cells (hPSCs) offer an invaluable model system for understanding the genetic basis of human cardiovascular diseases. Unlike nonhuman animal models, hPSCs can be tightly genetically matched to patients with disease. Previously, only in unusual circumstances was it feasible to study primary tissues such as cardiac muscle and blood vessels obtained directly from living patients, and even in such circumstances, the amount of tissue was limited. hPSCs represent a new approach that provides an unprecedented opportunity to study human cells that are matched to the patients of interest. Because they can be expanded into very large numbers and differentiated into a variety of cell types that are relevant to cardiovascular diseases—including cardiomyocytes, vascular endothelial and smooth muscle cells, and hepatocytes—hPSCs can in principle provide a limitless source of material with which to dissect the molecular underpinnings of the patient's disease process within and beyond the cardiovascular system (Figure 1).

hPSCs come in several varieties. Human embryonic stem cells (hESCs), first reported in 1998, are derived directly from human embryos.[1,2] Because making each hESC line typically entails the destruction of an embryo, the cell line is not matched to any living person. Arguably, this limits the utility of the hESC line for disease modeling and for precision medicine, because the health of the potential person represented by the source embryo is unknowable. Furthermore, most embryos used for the generation of hESCs have been left over from fertility treatments and, if implanted and taken to term normally, would not yield offspring expected to be strongly predisposed to any particular disease; however, as a result of preimplantation genetic diagnosis, in some cases the embryo is known to carry a specific, highly penetrant disease mutation. When the latter occurs, one can reasonably assume that the hESC line is a good proxy for the disease.

Representing a second type of hPSCs are stem cells derived by somatic cell nuclear transfer, which entails the transfer of a nucleus from a differentiated cell into a denucleated ovum.[3] Although this method is technically challenging and rarely used, it does offer a way to produce hPSC lines that are matched to people known to have the relevant disease. In 1 reported case, the hPSC line was made via somatic cell nuclear transfer from a patient with type 1 diabetes mellitus.[4]

The third type, induced pluripotent stem cells (iPSCs), is now the most abundant and widely used type of hPSCs. In their pioneering work, Yamanaka and colleagues explored the possibility of reprogramming somatic (ie, nongerm) cells into pluripotent stem cells via the heterologous expression of some combination of transcription factors.[5] They identified 4 factors (now known as the *reprogramming factors* or *Yamanaka factors*)—Oct3/4, Sox2, Klf4, and c-Myc—that could achieve the induction of pluripotency with mouse fibroblasts.[5] This discovery was followed by a rapid succession of studies reporting that the Yamanaka factors (or some variation thereof) could successfully reprogram various types of human somatic cells.[6–9]

Initially, there were concerns that despite also being pluripotent, iPSCs were substantively different than hESCs, which were considered the gold standard in the field. Over the past decade, these concerns have largely subsided. Although there is evidence that iPSCs initially retain some degree of epigenetic memory that reflects the identity of the somatic cell source, which could skew their ability to differentiate into certain cell types, this appears to dissipate as the cells are passaged in culture.[10–12] Initial reports suggested that the process of reprogramming itself resulted in aberrant epigenetic signatures in iPSCs that distinguished them from hESCs.[13,14] However, a recent study carefully examining this issue in isogenic (shared genetic background) hESCs and iPSCs concluded that transcriptional differences between hESCs and iPSCs are minor and inconsistent, and for all practical purposes, these cell types are molecularly and functionally equivalent.[15]

OBTAINING iPSCs FOR RESEARCH USE

The use of iPSCs has far exceeded the use of hESCs, because of the unique ability to genetically match iPSCs to living people for whom clinical data might be available. Moreover, hESCs have regulatory restrictions on their use. We outline the means by which investigators can obtain iPSCs from patients with specific traits or diseases, or iPSCs with specific genotypes of interest.

Methods to Derive New iPSC Lines

The first decision to make when generating iPSCs is the cell type to use for reprogramming. Many different somatic cell types have been demonstrated as being competent for the induction of pluripotency. Initially, dermal fibroblasts were a favored source; they could be readily obtained from a patient via a skin punch biopsy, a relatively noninvasive procedure, albeit a surgical procedure with a potential risk of infection of the biopsy site or (more likely) of the biopsied tissue itself. An important development in the field was the ability to perform reprogramming on peripheral blood cells, typically T cells.[16–18] Patients could be more easily recruited for a procedure that involves only a routine, nominally uncomfortable, sterile blood draw. It also made it feasible to draw upon peripheral blood cells that previously had been collected for unrelated purposes and placed in long-term storage in a frozen state. Other easily accessible, risk-free cellular sources for reprogramming are renal tubular cells collected from urine samples[19] and keratinocytes harvested from plucked hair follicles.[20] One consideration in choosing the cellular source is the concern that some types of cells, such as blood cells and dermal fibroblasts, might carry a higher mutational burden

because of high turnover rates and exposure to ultraviolet radiation. This could be particularly relevant when deriving iPSCs from older donors, because mutations and other abnormalities appear to occur with increasingly higher frequency with increased age.[21]

The second choice to make is the method by which to deliver the Yamanaka factors into the cell type. Initial efforts to generate iPSCs entailed the use of an integrating virus (eg, retrovirus or lentivirus) to introduce the factors as DNA transgenes. This led to concerns that constitutive expression of the factors would alter the biology of the iPSCs and interfere with their differentiation into somatic cell types, as well as the fear that undesirable transgenes could be inserted into the genome (eg, oncogenes or tumor suppressor genes). Indeed, the differentiation competency of iPSC lines could be affected by a disproportionate “stemness” load caused by integrated transgenes, which underscores the value of nonintegrative nuclear reprogramming.[22] Subsequent efforts focused on the use of transient, integration-free methods of delivering the Yamanaka factors, namely, via Sendai virus (a nonintegrating virus that eventually is lost from newly derived iPSCs as they are passaged),[23] episomal plasmid vectors,[24,25] minicircle vectors,[26] mini-intronic plasmids,[27] and messenger RNA transfection.[28] A recent comprehensive study comparing the various integration-free methods for the derivation of iPSCs concluded that there was no substantive difference in quality among the iPSC lines generated by the different techniques, but rather that the choice could be safely made based on the needs of the laboratory (Table 1).[29] For example, the authors endorsed the use of Sendai virus because of its efficacy in fibroblasts and blood cells, as well as its availability as a commercial reagent, but noted that the unavailability of clinical-grade Sendai virus makes it less useful in generating iPSCs intended for clinical use (eg, transplantation). RNA transfection was noted to be quick and efficient in fibroblasts but was not reported to be effective in blood cells. Episomal vectors were recommended for clinical-grade applications because of the simplicity of the reagents and quick clearance from iPSCs compared with the Sendai virus.

Upon successful generation of the desired iPSCs, various quality control measures are routinely undertaken. Traditionally, the gold standard to establish pluripotency is to implant the iPSCs into an immunodeficient animal and monitor for formation of teratomas that include tissues of all 3 developmental lineages (ectoderm, mesoderm, and endoderm). Because this is laborious and arguably entails the needless use of animals, newer alternative methods now include formation of embryoid bodies in vitro and assessment that they include cells of the 3 lineages; flow cytometry for markers of pluripotency, such as TRA-1–60 and SSEA-4; and detection of TRA-1–60 and SSEA-5 by nanoparticle-based surface-enhanced Raman scattering technology, which has been demonstrated to be highly sensitive and specific for the identification of pluripotent cells.[30] An additional check can be provided through the use of gene expression “scorecards” that evaluate the pluripotency or differentiation capacity of an iPSC line.[31,32]

Newly made iPSC lines should also be assessed for genomic integrity via cytogenetic karyotyping or array-based virtual karyotyping. The latter has the advantage of potentially detecting copy number changes and microdeletions and microduplications. There has been concern that iPSCs can accumulate mutations during the process of reprogramming,[33] which makes them less than perfectly matched to the donor individuals. Furthermore, other studies

have established that irrespective of reprogramming, hPSCs propagated for some length of time in culture will unavoidably accumulate mutations.[34–36] Routine exome or genome sequencing of newly generated iPSCs is not currently the norm, but as the cost of sequencing continues to fall, sequencing of new iPSC clones will be advisable to assess for any differences from the donor's germline genome. This might be especially warranted for iPSCs from older donors, in light of the observation that these iPSCs have a higher mutational burden at baseline.[21]

For iPSC lines that are intended for use for precision medicine applications (ie, to obtain information that will be directly translated to patient care, eg, response to a medication), even more stringent quality control measures are necessary. When they are first being established, iPSC lines should be monitored for clearance of whatever vectors were used for reprogramming (eg, episomal vectors, Sendai virus), and they should not be used until total clearance is rigorously confirmed. For as long as they are in use, iPSC lines need to be continuously monitored for infection, particularly with mycoplasma, which can alter the properties of the cells in ways that might confound phenotypic analyses. Periodic sequencing of iPSC lines as they are propagated in culture should be performed, both to confirm the identity of the cell lines (to ensure they have not inadvertently been mixed with or replaced by other cell lines in the laboratory) and to monitor for new mutations that might affect the properties of the cells. If the iPSC lines are to be used for differentiation into a particular cell type (eg, cardiomyocytes), the lines should initially be evaluated for their propensity to yield the desired cell type before they are committed to use in a study. Use of a poorly differentiating cell line will undermine the goals of the study. It should be recognized that these various quality control measures will add substantially to the costs of an iPSC-based study, and this needs to be proactively considered when planning the budget for the work.

Although it is quite feasible for reprogramming to be performed in an individual laboratory, a number of institutions have established core facilities dedicated to the generation, propagation, quality control, and dissemination of iPSC lines. This allows for a significant reduction of costs and streamlining of the procedure through bulk ordering of reagents and the efficient generation of multiple derivations in parallel by experienced staff. Core facilities now typically charge several thousand US dollars for the generation of iPSC lines from an individual donor, performed over the course of several months—a substantial cost and time investment, but given that only a decade has passed since the discovery of pluripotency induction, this represents remarkable progress.

Availability of Preexisting iPSC Lines

The easiest means by which an investigator can use iPSC lines from patients with specific diseases of interest, or individuals with particular genotypes of interest, is to obtain iPSC lines that have already been made elsewhere. Although this can be achieved via collaboration with other investigators, individual investigators will typically have made at most a few iPSC lines in their own laboratories, which limits the number of lines one can expect to obtain from collaborators. Drawing from the example set by the Jackson Laboratory in serving as a central repository to which investigators can contribute their

genetically modified mouse models and thereby make them available to the scientific community, several organizations have been seeking to establish so-called biobanks to serve as repositories for large iPSC collections that are easily accessible to any interested investigators.

In light of the considerable expense of generating iPSC lines, substantial funding is necessary to underwrite the establishment of biobanks. The California Institute of Regenerative Medicine (Oakland, CA) is underwriting the costs of generating 3 iPSC lines each from 3000 patients and healthy individuals, to be stored and distributed by the Coriell Institute for Medical Research (Camden, NJ). These iPSC lines are intended to be broadly representative of a variety of disorders, including cardiovascular diseases, liver diseases, respiratory diseases, Alzheimer disease, autism, and neurodevelopmental disabilities. The National Heart, Lung, and Blood Institute funded the Next Generation Genetic Association Studies (NextGen) Consortium, which is spread across 8 academic institutions in the United States with the goal of generating iPSC lines from >1500 individuals, including those with various cardiovascular, pulmonary, and blood disorders and their healthy control counterparts.[37] These cell lines are being stored and distributed by WiCell Research Institute (Madison, WI). On a smaller scale, the National Heart, Lung, and Blood Institute and the California Institute of Regenerative Medicine are funding the Stanford Cardiovascular Institute Biobank, which is generating iPSC lines from individuals with specific cardiovascular disorders such as hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), and cardiac rhythm disorders. Other regional efforts, including those sponsored through the Mayo Clinic Center for Regenerative Medicine and linked to dedicated Regenerative Medicine Consult Service portals, are under way to ensure long-term comprehensive repositories that cover a variety of human diseases. Similar international efforts are under way, such as the recently launched European Bank for induced pluripotent Stem Cells.

Genome-Editing Tools to Generate Stem Cell Lines With Specific Genotypes

In many cases, a particular disease-associated mutation might be of interest to an investigator but sufficiently rare in a population (and perhaps even unique to 1 individual) that it would be infeasible to recruit an individual with the mutation and generate iPSCs from that person without a relevant iPSC line being available in a biobank. In such cases, genome-editing tools can now introduce specific disease-associated mutations into normal or wild-type iPSC lines from healthy individuals (Figure 1). A variety of tools have been used for this purpose, including adenoviral vectors, zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems (Figure 2).[38] The recent introduction of CRISPR-Cas9 to edit mammalian cells has revolutionized the biomedical sciences because of its ease of use, allowing any investigator with basic molecular biology skills to use it, and because of its relatively high efficiency compared with other tools.

Regardless of whether zinc-finger nucleases, TALENs, CRISPR-Cas9, or even newer nucleases are used, the aim is to introduce double-strand DNA breaks at desired locations in the genome. The cellular DNA repair machinery uses 1 of 2 mechanisms to restore the

integrity of a site of a DNA break, nonhomologous end-joining or homology-directed repair (HDR) (Figure 2). Nonhomologous end-joining is an error-prone process that can introduce insertions or deletions (indels) of various sizes, although such indels are usually a few base pairs in length. HDR is a more precise repair mechanism that uses a homologous template—typically a sister chromatid or chromosome, but alternatively a synthetic, custommade template introduced into the cell by the investigator, allowing for the introduction of specific, desired changes into the genome. In principle, genome editing via HDR provides a means by which any desired disease-associated mutation can be introduced into any iPSC line (or, conversely, a preexisting mutation can be corrected). It can also be used to introduce useful DNA sequences, such as a reporter gene in a desired locus. However, HDR is typically a less efficient mode of genome editing than nonhomologous end-joining, because HDR is restricted to the S and G2 phases of the cell cycle, whereas nonhomologous end-joining occurs throughout the cell cycle. Accordingly, it is easier to knock out genes by introducing frameshift indels into coding sequences than it is to knock in a mutation.

The ability to introduce desired mutations into iPSC lines has several potential advantages compared with generating iPSC lines from patients. Except in cases where a patient with the desired mutation is simply not accessible to the investigator, it will typically be much quicker and less expensive to use genome editing than it is to recruit and consent a new patient into a research study, collect somatic cells, undertake reprogramming, and perform quality control and then expansion of iPSC clones through a number of passages before they can be used for studies. The investigator can genome-edit a well-characterized wild-type cell line that has been used for studies previously and for which differentiation protocols have already been tested and optimized rather than take the chance that the newly generated patient-specific iPSC lines may not prove suitable for the desired studies. However, there is a potential shortcoming to this shortcut approach, because it depends on the disease-associated mutation being fully penetrant in the chosen wild-type iPSC line. If the iPSC line does not have a permissive genetic background (ie, if it contains genetic modifiers that mitigate the phenotypic consequences of the mutation), then the genome-edited iPSC line will not properly reflect the disease state. This is presumably not an issue with a patient-derived iPSC line, because the very fact of the patient having the disease indicates that the genetic background is permissive for the phenotypic consequences of the mutation.

Yet another major advantage of genome editing of iPSCs is that it allows more rigorous study designs than the generation of iPSC lines from genetically unmatched disease patients and control individuals, an issue that is discussed in more detail in Study Design.

iPSCs FOR CARDIOVASCULAR DISEASE MODELING

Disease modeling represents perhaps the most productive use of iPSCs to date. A singular advantage of iPSCs is that they are genetically matched to the person from whom they were derived, which makes them ideally suited for the study of diseases that have a strong underlying genetic cause, such as classic monogenic disorders. At the same time, the fact that iPSCs have been reprogrammed from adult cells into a baseline state stripped of many of the epigenetic changes caused by environmental influences during the person’s lifetime allows the investigator to better study the genetic contribution to the disease. This is an

important consideration when modeling complex diseases that typically reflect the interplay of multiple genetic and environmental factors. In either case, iPSCs have proven useful in helping researchers better understand how disease genotypes at the genetic level are manifested as phenotypes at the cellular level with respect to the broad disease categories described in this section (Table 2).[39]

Cardiomyopathies

Familial cardiomyopathies are among the best characterized of monogenic cardiovascular disorders, with mutations in dozens of genes having been linked to various forms of cardiomyopathy. As such, there has been significant interest in the generation of iPSCs from patients with familial cardiomyopathies and differentiation of the iPSCs into cardiomyocytes (referred to here as iPSC-CMs), as well as early success in gaining new molecular insights into the pathogenesis of the disorders.

DCM is the most common type of cardiomyopathy, with familial DCM cases thought to represent a substantial proportion of the total cases. DCM manifests with ventricular enlargement and thinning and heart failure from a severely compromised ejection fraction. The most commonly mutated gene in familial DCM cases is *TTN* (titin), followed by *LMNA* (prelamin-A/C), *MYH7* and *MYH6* (myosin-7 and −6), *SCN5A* (sodium channel protein type 5 subunit alpha), *MYBPC3* (myosin-binding protein C, cardiac-type), and *TNNT2* (troponin T, cardiac muscle).[40] In almost all cases, familial DCM is associated with dominant mutations. Perhaps 10% to 20% of familial DCM cases can be attributed to truncating mutations (frameshift, nonsense, or splice site) in *TTN*, which encodes a component of the sarcomere.[41] iPSCs generated from DCM patients with either truncating or missense mutations in *TTN*, when differentiated into iPSC-CMs and assembled into cardiac microtissues, displayed sarcomere insufficiency, impaired responses to mechanical and β-adrenergic stress, and attenuated growth factor and cell signaling activation.[42] Genome-edited wild-type iPSC-CMs into which *TTN* truncating mutations had been introduced displayed similar phenotypes.[42]

The most intensively studied familial DCM iPSC lines to date were derived from a family whose affected members harbor a missense R173W mutation in *TNNT2*, which also encodes a component of the sarcomere. Several iPSC lines were generated from affected family members, and several were generated from unaffected family members as control lines. The mutant iPSC-CMs exhibited abnormal calcium handling, reduced contractility, and myofibrillar disarray, which were exacerbated with β-adrenergic stimulation.[43] A deeper molecular characterization of these cell lines revealed increased expression of the phosphodiesterase genes *PDE2A* and *PDE3A* via epigenetic activation that was responsible for the compromised β-adrenergic signaling and contractile dysfunction.[44]

A number of other studies have characterized iPSC-CMs from patients with DCM, either from a primary familial disorder or as part of a genetic syndrome. iPSC-CMs with either a nonsense or missense mutation in *LMNA* were found to have increased nuclear bleb formation and micronucleation, as well as increased apoptosis on electrical stimulation.[45] iPSC-CMs with an in-frame deletion mutation in *PLN* (phospholamban) showed calcium handling abnormalities that were reversed in iPSC-CMs in which the mutation had been

corrected by genome editing.[46] A novel *DES* (desmin) missense mutation found by exome sequencing in a patient with DCM was associated with abnormal desmin aggregations, calcium handling, and response to inotropic stress in the patient's iPSC-CMs.[47] Duchenne muscular dystrophy is a primarily skeletal muscle disorder that is often accompanied by a dilated-type cardiomyopathy, which is often the underlying cause of death in older children surviving past the initial (skeletal-based) stages of the disease. iPSC-CMs from a number of patients with Duchenne muscular dystrophy displayed abnormalities consistent with this disease pathophysiology.[48,49] Barth syndrome is a mitochondrial disorder cause by *TAZ* (tafazzin) mutations that affects both cardiac and skeletal muscle and manifests in part as DCM. iPSC-CMs from 2 patients with either a frame-shift or missense mutation in *TAZ* exhibited mitochondrial defects, excess levels of reactive oxygen species, abnormal sarcomere assembly, and impaired contractility.[50] Genome-edited wild-type iPSC-CMs into which *TAZ* frameshift mutations were introduced had the same abnormalities as the patient-specific iPSC-CMs.[50]

Familial HCM is a dominant disorder that manifests as asymmetrical ventricular wall thickening with increased risk of sudden cardiac death (SCD). The most commonly mutated genes in familial HCM cases are sarcomere components, including *MYH7*, *MYBPC3* (myosin-binding protein C, cardiac type), *TNNT2*, *TNNI3* (troponin I, cardiac muscle), and *TPM1* (tropomyosin α−1 chain).[51] The most intensively studied familial HCM iPSC lines to date were derived from a family whose affected members harbor a missense mutation in *MYH7*. The iPSC lines were generated from 10 affected and unaffected family members (2 parents and 8 children).[52] Besides displaying sarcomere disarray, the mutant iPSC-CMs displayed cellular enlargement and contractile arrhythmia in the setting of abnormal calcium handling, both of which could be normalized by treatment with the calcium channel blocker verapamil. iPSC-CMs with a different *MYH7* missense mutation also displayed sarcomere disarray and electrophysiological abnormalities that could be normalized with verapamil treatment.[53]

Arrhythmogenic right ventricular cardiomyopathy (ARVC), also called arrhythmogenic right ventricular dysplasia, is typically a dominant disorder in which there is gradual, adult-onset fibrofatty replacement of cardiomyocytes, predominantly in the right ventricle but sometimes affecting the left ventricle, with increased risk of SCD. The most commonly mutated genes in ARVC cases are components of a structure involved in cell-to-cell adhesion called desmosome: *PKP2* (plakophilin 2), *DSG2* (desmoglein 2), *DSP* (desmoplakin), *DSC2* (desmocollin 2), and *JUP* (junction plakoglobin).[54] The most intensively studied ARVC iPSC lines to date were derived from 2 unrelated individuals, one homozygous for a mutation in *PKP2* that causes a splicing defect and the other heterozygous for a frame-shift mutation in *PKP2*.[55] Although the iPSC-CMs from these patients exhibited subtle molecular defects in standard differentiation conditions, they did not fully manifest ARVC-related phenotypes until they were exposed to conditions that resulted in metabolic aging, namely, treatment with a cocktail of 5 adipogenic factors, whereupon they displayed increased lipogenesis and apoptosis, as well as abnormal calcium handling. In another study, iPSC-CMs from 2 patients heterozygous for different *PKP2* frameshift mutations had increased lipid accumulation and desmosomal distortion in standard differentiation conditions, phenotypes that were intensified on treatment with adipogenic medium.[56] These

same findings were observed in yet another study with iPSC-CMs from a patient heterozygous for a missense mutation in *PKP2*.[57] Phenotypes observed in iPSC-CMs from patients with *PKP2* mutations could be reversed with a small molecule originally identified in a high-throughput screen in a zebrafish model of ARVC.[58] A lesson to draw from these studies is that extra stimuli could be needed to fully provoke disease-related phenotypes in the in vitro differentiated cardiomyocytes, particularly with adult-onset diseases such as ARVC. However, caution also must be taken, because the relevance of the extra stimuli to the native human myocardium in the disease setting might be unclear. In addition, these studies also suggest the need to perform more comprehensive side-by-side comparisons between iPSC-CM phenotypes and cardiac abnormalities found in actual donor hearts.

Left ventricular noncompaction, also known as spongiform cardiomyopathy, results when trabeculated structures in the ventricular wall that form during development fail to undergo typical compaction from spongy into solid structures.[59] Potential clinical consequences include heart failure and SCD. iPSC-CMs from family members with left ventricular noncompaction who harbored a nonsense mutation in *TBX20* (T-box protein 20), compared with iPSC-CMs from unaffected family members, displayed a proliferation defect and abnormal activation of transforming growth factor-β signaling, a finding that was also observed in genetically modified mice.[60] iPSC-CMs from patients with delayed-onset cardiomyopathy with noncompaction linked to a missense mutation in *GATA4* (GATA binding protein 4) showed impairments in contractility, calcium handling, and metabolic activity.[61]

Rhythm Disorders

Arrhythmic syndromes are the cardiovascular disorders most extensively studied with iPSC-based models to date, in particular the long-QT syndromes (LQTS). LQTS is marked by delayed repolarization of the heart after contraction, which manifests as an increased QT interval on the ECG and predisposes patients to ventricular arrhythmias and SCD. LQTS is usually inherited in an autosomal dominant manner, although there are a few forms that are inherited in an autosomal recessive manner. Mutations in at least 15 genes have been linked to LQTS, typically affecting the function of potassium, sodium, or calcium channels in cardiomyocytes.[62] The 3 most commonly implicated genes, respectively, responsible for LQTS1, LQTS2, and LQTS3, are *KCNQ1* (potassium voltage-gated channel subfamily Q member 1), *KCNH2* (potassium voltage-gated channel subfamily H member 2, also known as hERG), and *SCNA5* (sodium voltage-gated channel α-subunit 5). Intriguingly, these 3 subtypes of LQTS appear to have different triggers for ventricular arrhythmia.[63] LQTS1 is triggered by exercise, particularly swimming, whereas LQTS2 is triggered by emotional stress and auditory stimuli (such as alarm clocks). In contrast, arrhythmic events in LQTS3 tend to occur during sleep. These distinct triggers suggest distinct mechanisms by which mutant alleles of the 3 genes predispose to SCD.

In the first published iPSC-based study of an arrhythmic syndrome, iPSCs were derived from affected members of a family with autosomal dominant LQTS1 associated with a missense mutation in *KCNQ1*.[64] Mutant iPSC-CMs displayed prolongation of the action potential duration (APD), as well as increased tachyarrhythmia on exposure to a β-

adrenergic agonist, isoproterenol. The mutation was found to confer a dominant-negative impairment of membrane localization of the KCNQ1 channel subunit, resulting in a 70% to 80% reduction in the slowly activating delayed rectifier potassium current (I_{Ks}) in the iPSC-CMs. Subsequent studies of iPSC-CMs from LQTS1 patients with *KCNQ1* missense or frameshift mutations found similar phenotypes.[65,66] iPSC-CMs generated from a patient with Jervell and Lange-Nielsen syndrome, a recessive disorder with severe QT prolongation, congenital bilateral deafness, and high risk of SCD that is usually caused by mutations in *KCNQ1*, showed the expected pronounced changes in APD, I_{Ks} current, and sensitivity to proarrhythmic stressors, as did iPSC-CMs in which genome editing was used to introduce *KCNQ1* mutations.[67]

Numerous studies with iPSC-CMs from patients with LQTS2 associated with missense mutations in *KCNH2* have reproducibly shown increased APDs, increased arrhythmogenicity, and decreased rapidly activating delayed rectifier potassium current (I_{Kr}), which is sometimes linked to defective trafficking of the hERG protein.[68–73] In one study of LQTS2 iPSC-CMs, genetic correction of the *KCNH2* mutation reversed the phenotypes,[71] and in another study, specific knockdown of the mutant *KCNH2* allele with RNA interference reversed the phenotypes, establishing it as a dominant-negative allele.[74] In a similar fashion, iPSC-CMs from patients with LQTS3 associated with *SCN5A* mutations[75,76] or Timothy syndrome associated with mutations in *CAC-NA1C* (calcium voltage-gated channel subunit-αC)[77] were found to have increased APDs and either abnormal sodium currents or an abnormal calcium current. Of note, both LQTS1 and LQTS2 have been modeled with iPSC-CMs in which mutant genes were inserted with genome editing, which avoids the need to recruit patients with the particular mutations and to generate new iPSC lines for each patient.[78]

Another rhythm disorder that has proven amenable to modeling with iPSC-CMs is catecholaminergic polymorphic ventricular tachycardia (CPVT). In CPVT, ventricular arrhythmias are triggered by exercise or emotional stresses in the absence of structural heart disease. Although these arrhythmias are often self-resolving, SCD does occur in many CPVT patients. The most common cause of CPVT is autosomal dominant mutations in *RYR2* (ryanodine receptor 2), with the next most common being autosomal recessive mutations in *CASQ2* (calsequestrin 2); both genes encode regulators of calcium storage and release in the sarcoplasmic reticulum in cardiomyocytes.[79] A large number of studies of iPSC-CMs from CPVT patients with missense mutations in one or the other gene have documented abnormal calcium handling and increased arrhythmogenicity on adrenergic stimulation.[80–87]

Brugada syndrome is yet another disorder that has been modeled with iPSC-CMs. Brugada syndrome, which like LQTS3 has been linked to mutations in *SCN5A* but is quite distinct from LQTS3, is a disorder that is associated with characteristic electrocardiographic precordial ST-segment elevation and predisposes to ventricular fibrillation and SCD. iPSC-CMs from patients with Brugada syndrome displayed blunted inward sodium currents, increased triggered activity, and abnormal calcium handling; these cellular phenotypes were rescued with genome editing to correct the causal *SCN5A* mutation.[88]

Valvular and Vascular Disorders

Bicuspid aortic valve with calcification of the valve is the most common congenital valvular disorder. A familial form of the disease with severe valve calcification has been linked to heterozygous nonsense mutations in *NOTCH1*, which encodes a transcriptional regulator.[89] iPSCs from patients with *NOTCH1* mutations were subjected to genome editing to correct the mutations, followed by differentiation of the isogenic cell lines into endothelial cells.[90] When assessed in an in vitro model of vascular shear stress, the mutant iPSC-endothelial cells failed to activate antiosteogenic, anti-inflammatory, and antioxidant pathways that were induced in control iPSC-endothelial cells, consistent with a predisposition towards calcification of the aortic valve.

Supravalvular aortic stenosis can occur in isolation as the result of mutations in *ELN* (elastin) or as part of Williams-Beuren syndrome, in which a deletion on chromosome 7q11.23 entirely removes the *ELN* gene.[91] iPSCs from a patient with a frameshift mutation in *ELN*, after differentiation into smooth muscle cells, had defective actin filament bundle formation, a significantly higher proliferation rate, and a higher migration rate in a chemotaxis assay.[92] Similar phenotypes, along with impaired vascular tube formation, were observed in iPSC-smooth muscle cells from Williams-Beuren patients,[92,93] and the phenotypes could be normalized with treatment with the drug rapamycin.[93]

Pulmonary arterial hypertension causes progressive right-sided heart failure that is accompanied by substantial morbidity and mortality. A subset of patients with pulmonary arterial hypertension exhibit an inherited form of the disease, with the majority of familial cases linked to mutations in *BMPR2* (bone morphogenetic protein receptor 2). iPSC-endothelial cells from patients with familial pulmonary arterial hypertension and *BMPR2* mutations displayed reduced adhesion, survival, migration, and angiogenesis compared with iPSC-endothelial cells from either unaffected carriers of *BMPR2* mutations or healthy individuals without *BMPR2* mutations.[94] These findings suggest that iPSC-endothelial cells can model not only familial pulmonary arterial hypertension caused by *BMPR2* mutations but also the effects of genetic modifiers of *BMPR2* mutations resulting in an absence of disease.

Metabolic Risk Factors for Ischemic Heart Disease

Blood concentrations of lipids, namely, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol, are well-established risk factors for ischemic heart disease. A number of monogenic lipid disorders have been defined. The best known is autosomal dominant hypercholesterolemia, also known as familial hypercholesterolemia (FH), which can be caused by mutations in *LDLR* (LDL receptor), *APOB* (apolipoprotein B), or *PCSK9* (proprotein convertase subtilisin/kexin type 9), all of which are important regulators of blood LDL-C levels.[95] Two studies generated iPSCs from FH patients with mutations in *LDLR* and found that differentiated hepatocytes were impaired in LDL particle uptake, consistent with the elevated blood LDL-C levels seen in FH patients.[96,97] The second study also found that the patient-derived hepatocytes responded to treatment with a statin drug that increased LDL uptake.[97] Another study generated iPSCs from an FH patient with a gain-of-function mutation in *PCSK9* and from a patient with familial

hypobetalipoproteinemia who had an abnormally low LDL-C level caused by a loss-of-function *PCSK9* mutation. As with the *LDLR* mutant cell lines, the FH iPSC-hepatocytes displayed impaired LDL particle uptake, whereas the familial hypobetalipoproteinemia iPSC-hepatocytes displayed increased LDL particle uptake.[98]

Tangier disease, also known as hypoalphalipoproteinemia, is marked by abnormally low blood levels of high-density lipoprotein cholesterol caused by recessive mutations in *ABCA1* (ATP-binding cassette subfamily A member 1). ABCA1 is the molecule that effluxes cholesterol from cells such as macrophages onto high-density lipoprotein particles and thus is a key mediator of reverse cholesterol transport. iPSCs derived from patients with Tangier disease with missense or nonsense *ABCA1* mutations displayed impaired cholesterol efflux on differentiation into macrophages.[99] The patient iPSC-macrophages also showed increased expression of proinflammatory cytokines. In a complementary study, genome editing was used to introduce frameshift mutations in the *ABCA1* gene to knock out the gene in hPSCs.[100] As with the aforementioned Tangier disease patient iPSC-macrophages, *ABCA1*-knockout differentiated macrophages had both impaired cholesterol efflux and increased expression of cytokines compared with isogenic wild-type differentiated macrophages.

Stem cell–based analyses proved useful in clarifying the function of *SORT1*, a novel regulator of blood LDL-C levels identified by genome-wide association studies. Conflicting studies in mice disagreed on whether hepatic *SORT1* decreased or increased lipoprotein particle secretion.[101,102] Genome editing was used to knock out the *SORT1* gene in hESCs, and *SORT1*-knockout differentiated hepatocytes displayed increased lipoprotein particle secretion.[103] *SORT1* also has a role in insulin-stimulated glucose transport, confirmed by the finding that glucose transport was unchanged in *SORT1*-knockout differentiated adipocytes on stimulation with insulin, in contrast to isogenic wild-type differentiated adipocytes, in which glucose transport was increased.[103]

Type 2 diabetes mellitus and its attendant insulin resistance are also risk factors for ischemic heart disease. As with other diseases, iPSCs have proven the most useful when modeling monogenic forms of diabetes mellitus. iPSCs derived from patients with maturity-onset diabetes of the young type 2, caused by missense mutations in *GCK* (glucokinase), were differentiated into pancreatic β-cells and transplanted into mice, whereupon they were found to require higher blood glucose levels to stimulate insulin secretion than control iPSC-derived β-cells.[104] This phenotype was reversed when genome editing was used to correct the *GCK* missense mutations.

A dominant negative missense mutation in *AKT2* (V-Akt murine thymoma viral oncogene homolog 2) was found to be responsible for severe insulin resistance and diabetes mellitus,[105] whereas a putative gain-of-function missense mutation in *AKT2* resulted in hypoinsulinemic hypoglycemia and hemihypertrophy, with clinical signs opposite to those of diabetes mellitus.[106] Both of these conditions were modeled in an isogenic series of genome-edited hESCs, without the need to recruit the rare patients with these mutations: insulin resistance via knockout of the *AKT2* gene, and hypoinsulinemic hypoglycemia via knockin of the *AKT2* gain-of-function missense mutation.[103] *AKT2*-knockout-hepatocytes

displayed increased glucose production in response to stimulation with dexamethasone, forskolin, and insulin, whereas *AKT2*-knockin hESC-hepatocytes displayed decreased glucose production. *AKT2*-knockout hESC-adipocytes showed impaired insulin-stimulated glucose transport and decreased lipid accumulation, whereas *AKT2*-knockin hESC-adipocytes showed increased baseline glucose transport, regardless of the presence of insulin, and increased lipid accumulation. These in vitro phenotypes accord well with the metabolic disturbances of the patients. Additionally, *AKT2*-knockin hESC-adipocytes were observed to have abnormally high secretion of inflammatory molecules, which suggests an unrecognized inflammatory component to hypoinsulinemic hypoglycemia and hemihypertrophy.[103]

METHODOLOGICAL CONSIDERATIONS IN DISEASE MODELING

Study Design

The vast majority of iPSC-based disease-modeling studies have compared 1 or only a few iPSC lines from patients with a disease with 1 or a few iPSC lines from healthy control individuals. In general, these studies have concluded that the phenotypic differences observed between differentiated disease cells and differentiated control cells are attributable to the disease mutations. However, such conclusions are potentially confounded by other factors that can underlie the observed differences. Most obviously, differences in genetic background are inevitable unless the comparators are identical twins (in which case one would not expect to see any phenotypic differences between the iPSC lines in the first place). Such differences are a particular concern when the disease cells and the control cells are not matched for sex and ethnicity. Some studies attempted to mitigate the confounding by using control cells from unaffected individuals in the same families as the patients from whom the disease cells were derived. Nonetheless, because only ≈50% of the genome is shared between any 2 first-degree relatives, confounding from the genetic differences in the other ≈50% of the genome is still a possibility.

Even if the subjects from whom the iPSCs were derived are well matched, there are a host of other potential confounders that could complicate the conclusions of a study. Factors such as differences in the cellular sources for the iPSCs (eg, skin versus blood versus urine), differences in the methodologies used for the induction of pluripotency (eg, lentivirus versus Sendai virus versus episomes versus RNA transfection), differences in the epigenetic states of the iPSC lines used, differences in the passage numbers and acclimation to cell culture conditions of the iPSC lines, and differences in the capacity for differentiation to the desired cell type all can come into play.

With so many potential confounding factors, studies with unmatched iPSC lines should take steps to minimize their influence. One consideration is the expected effect of the disease-associated mutations under study. If the disease is a dominant monogenic disorder, and the disease mutations are presumed to be highly penetrant and have outsize phenotypic effects, then the signal that results from a mutation might well outweigh the noise that results from the confounding factors. However, if a complex, polygenic disease is being studied, any given disease-associated variant could have a small effect size that is swamped by the confounders. This problem is exacerbated if the disease under study is only partly heritable

or very heterogeneous, or if the responsible disease-associated variants are unknown. In such cases, the study would be best served by using large numbers of disease and control iPSC lines so as to balance out the confounding factors and thus sharpen the signal-to-noise ratio. However, it can be challenging to use a large number of lines because of patient scarcity, expense of generating the lines, laboratory capacity, etc. The growing number of biobanks from which many iPSC lines for a particular rare disease are available (eg, HCM) or through which a genetically diverse population cohort of iPSC lines is accessible (to make it possible to identify many lines with a particular common DNA variant) now make it more feasible to conduct large, well-powered studies. One caveat is that many iPSCs derived from patients categorized as “diseased” could be from patients who fall under a variable spectrum of disease (especially in complex forms) and not necessarily end stage; thus, careful side-by-side analysis of iPSC phenotypes to donor tissues would be necessary to determine whether this clinical heterogeneity can be recapitulated in iPSCs. However, if true, this can be advantageous in identifying early drivers of disease.

The recent emergence of efficient genome-editing tools provides alternative study designs that eliminate most of the aforementioned confounders. For instance, genome editing can be used to introduce a disease-associated mutation into a wild-type iPSC line, yielding isogenic cell lines that are matched for origin, acclimation to culture conditions, epigenetic status, differentiation capacity, etc. Any differences observed between wild-type and mutant iPSC lines can be more reliably attributed to the mutation itself, thus establishing a causal connection between genotype and phenotype. However, as mentioned in Genome-Editing Tools, this study design may not be informative if the mutation being modeled is not highly penetrant with a large effect size and if the genetic background of the chosen iPSC line is less permissive (protective) for the disease. Put another way, this study design tests for the sufficiency of the mutation to cause disease phenotypes, regardless of genetic background (Figure 3).

A different study design entails genome editing of a patient-specific iPSC line to correct the disease-associated mutation. This is somewhat different from introducing the mutation into a wild-type iPSC line, because it tests for the necessity of the mutation to cause disease phenotypes and does not presume that the mutation is highly penetrant (Figure 3). Because the iPSC line was derived from a patient with the disease, it can be presumed that the genetic background is permissive for the disease. In principle, the most rigorous disease-modeling studies would be those in which genome editing of both wild-type and disease iPSCs is performed to test the sufficiency and necessity of a disease mutation in parallel.

The major shortcoming of the genome-editing approach is that it relies on the investigator having properly identified a disease-associated variant to study. If the genetic cause of a disease remains unclear but the disease clearly segregates within a family in a monogenic fashion, then the use of iPSCs from family members might be more productive in learning about the molecular underpinnings of a disease. If the disease is complex (ie, polygenic), then genome editing might not be informative, because numerous variants would need to be edited to fully reproduce (or fully eliminate) the disease state. Genome editing remains sufficiently arduous that altering >1 variant at a time is technically prohibitive, and editing a single variant might not be instructive because of its relatively small contribution to disease.

In addressing a complex disease, use of a large number of well-matched patient and control iPSC lines might instead be the most viable approach.

Finally, it is worth noting a study design that is unmoored from any particular disease but that instead seeks to better understand the molecular consequences of naturally occurring genetic variations. In principle, the use of a genetically diverse population cohort of iPSCs numbering in the dozens to hundreds will have sufficient representation of alternate alleles of common genetic variants to identify associations between genotypes and expression levels of nearby genes, known as expression quantitative trait loci (eQTLs) (Figure 4). To date, eQTL studies have been performed with primary tissues obtained from living patients who have undergone surgery or from postmortem cadaveric donors. For obvious reasons, these might not reflect the highest-quality tissues; furthermore, the intact tissues comprise an admixture of multiple cell types, and the obtained material is nonrenewable. By contrast, iPSCs offer an inexhaustible supply of differentiated cells of a single type for use in eQTL studies. Such studies could yield new insights not only into the tissue-specific regulation of gene expression but possibly into other molecular mechanisms, such as the regulation of RNA transcript splicing. One recent study used a population cohort of iPSC-hepatocytes to perform eQTL studies and discovered several novel causal variants and causal genes involved in lipid metabolism.[107]

Differentiation Protocols

An enormously attractive property of iPSCs is that they can be differentiated in vitro into a variety of lineages— in theory, into all of the existing cell types in the human body. The previous section on cardiovascular disease modeling described studies that used the following differentiated cell types to assess disease-related phenotypes: cardiomyocytes, endothelial cells, smooth muscle cells, hepatocytes, macrophages, and adipocytes. Although such studies can prove informative, they can also have the limitations inherent in the differentiation protocols, namely, the lack of consistency, lack of purity, and lack of maturity of the differentiated cells.

This is illustrated by the continuing efforts to develop and optimize protocols to yield pure, mature cardiomyocytes from hPSCs (Figure 5). Initial protocols involved the dispersion of hPSCs and culturing in suspension, followed by spontaneous aggregation of the cells into embryoid bodies. As a result of spontaneous differentiation of cells in the embryoid bodies into different lineages, some proportion of the cells take on the properties of cardiomyocytes and can then be isolated and studied.[108,109] iPSCs have proven to be just as amenable to embryoid body–based differentiation as hESCs.[110,111] However, embryoid body–based differentiation is labor intensive and has low efficiency with respect to the proportion of functional cardiomyocytes, as well as significant sample-to-sample variability. This has prompted exploration of protocols in which hPSCs are differentiated in a 2-dimensional monolayer format, which greatly simplifies the process.[112] An important conceptual innovation was the use of growth factors or protein inhibitors to direct the differentiation of hPSCs into cardiomyocytes, rather than relying on the hPSCs to spontaneously differentiate into cardiomyocytes.[112–116] A widely used monolayer-based differentiation protocol incorporates glycogen synthase kinase-3β and Wnt inhibitors, yielding cardiomyocytes with

an efficiency as high as >90%.[117] A more recent protocol simplifies the process even further by combining glycogen synthase kinase-3β and Wnt inhibitors with a 3-component basal medium (as opposed to previous protocols that used a basal medium with >20 components).[118] In principle, simplification of differentiation conditions should foster more sample-to-sample consistency and reproducibility.

Another approach to generating differentiated cardiomyocytes in large numbers is to convert hPSCs into multipotent cardiac progenitor cells (CPCs) that in turn have the capacity to be differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells.[113,119,120] CPCs have the ability to proliferate in culture, which in principle makes it possible to greatly expand a CPC source from which cardiomyocytes (and the 2 other cell types) can then be made at large scale. Exploration of directed differentiation conditions that permit the conversion of CPCs into a pure population of cardiomyocytes, rather than a mix of 3 cell types, is a work in progress. The recent demonstration of the ability to directly reprogram mouse fibroblasts into expandable CPCs,[121,122] without the need for an intermediate iPSC stage, points to the possibility of using human “iCPCs” instead of iPSCs for disease modeling and precision medicine studies in the future, although rigorous quality control measures for the CPCs would need to be established.

Despite these advances in cardiomyocyte differentiation, the lack of purity and lack of maturity remain obstacles. For some studies, mixed populations of cardiomyocytes and noncardiomyocytes might be adequate, as in cases when one can visually inspect for spontaneously beating cells and focus one’s analyses on just those cells. However, more generally, highly pure preparations of cardiomyocytes are desirable and optimal. One strategy is to use a cardiomyocyte-specific membrane protein with which to deploy antibodies for fluorescence-activated cell sorting or magnetic-activated cell sorting to separate cardiomyocytes from other cells, followed by replating of the purified population of cardiomyocytes. Signal-regulatory protein-α (SIRPA) and vascular cell adhesion molecule 1 (VCAM1) have been identified as 2 such cardiomyocyte markers, and cell sorting with antibodies against either of these proteins resulted in >95% pure populations of functional cardiomyocytes.[123,124] A fluorescent dye that labels mitochondria and tetramethylrhodamine methyl ester perchlorate can be similarly used to sort cardiomyocytes from noncardiomyocytes.[125] However, a disadvantage to this strategy is that it requires additional manipulation of the cells, namely, dispersion from the culture dish followed by sorting through a machine and then replating. Not only is this labor intensive, especially when working with a large number of samples, it also can adversely affect the quality of the cells.

An ideal approach would be to purify the cardiomyocytes while still in the dish. In one study, lentiviral vectors were used to introduce antibiotic resistance genes driven by cardiomyocyte-specific promoters into hPSCs.[126] In a test case, drug selection in the dish after differentiation of infected cells yielded 96% pure cardiomyocytes. In another study, an antibiotic resistance gene was inserted by homologous recombination directly into the endogenous *MYH6* locus such that the gene would be coexpressed with *MYH6* in cardiomyocytes, with drug selection resulting in >98% purity.[127] In yet another study with a more sophisticated approach, genome editing of hPSCs was used to incorporate a quiescent

transgene harboring an antibiotic resistance gene.[128] Differentiated cells were treated with a lentivirus expressing Cre recombinase from a muscle-specific promoter to potentiate the antibiotic resistant gene only in cardiomyocytes. Drug selection in the dish improved the yield of cardiomyocytes from 80% to >99%. A nongenetic strategy to purify iPSC-CMs in the dish takes advantage of their ability to metabolize lactate, in contrast to undifferentiated cells; prolonged incubation with glucose-depleted, lactate-rich medium increases the yield of iPSC-CMs to as high as 99%.[129] However, even when purity is achieved with any of these strategies, heterogeneity remains in the iPSC-CMs; differentiated cardiomyocytes are typically a mixture of cells, some of which most closely resemble ventricular cells, with others resembling atrial cells and still others resembling nodal cells.[109] Obtaining a pure population of just 1 of these cardiomyocyte subtypes is a challenge that remains to be solved, although some progress has been made.[114,130–135] For example, a recently reported protocol can generate nodal-like cells of >80% purity.[135]

Although the strategies outlined above can successfully purify in vitro differentiated iPSC-CMs, the cells display important differences from authentic adult human cardiomyocytes and are more similar to fetal human cardiomyocytes (Table 3).[136–139] Whereas adult cardiomyocytes are long and cylindrical in shape, differentiated cells are more rounded and much shorter, with a smaller length-to-width ratio.[140] The adult cells are multinucleated, whereas differentiated cells tend to be mononuclear, like fetal cardiomyocytes.[141] Furthermore, differentiated cells have less well-developed sarcoplasmic reticulum and lack transverse tubules, which results in calcium handling that relies more on flux through the sarcolemma rather than the sarcoplasmic reticulum, which resembles fetal cardiomyocytes.[142,143] With respect to metabolism, iPSC-CMs have fewer mitochondria and accordingly depend more on glycolysis rather than β-oxidation of fatty acids for energy.[55,125,144] In general, iPSC-CMs have higher resting membrane potentials (ranging from −30 to −75 mV) than adult ventricular cardiomyocytes (−85 to −90 mV) but slower depolarization speeds.[136,137] The gene expression profile of differentiated cells most closely resembles that of fetal cardiomyocytes and is quite distinct from that of adult cardiomyocytes with respect to calcium handling and cardiac ion channel genes.[145–147]

A number of strategies have been attempted to increase the maturity of iPSC-CMs and make them more similar to adult cardiomyocytes, although a definitive solution to the problem remains a vigorously pursued goal in the field. One strategy has been to simply maintain the cells for a lengthier time in culture. Compared with iPSC-CMs differentiated and maintained for 20 to 40 days, cells maintained for 80 to 120 days were increased in size and anisotropy and had greater myofibril density and alignment, visible sarcomeres, a much larger proportion of multinucleated cells, hyperpolarized maximum diastolic potentials, increased action potential amplitudes, and faster upstroke velocities.[148] In a separate study, iPSC-CMs maintained for a full year accrued structural characteristics of mature cells.[149]

Altering the stiffness or morphology of the growth substrate for iPSC-CMs is an increasingly popular approach. One study found that differentiated cardiomyocytes maintained on polyacrylamide hydrogels with increasing degrees of stiffness attained morphologies more similar to adult cells.[150] Another study found that iPSC-CMs maintained on stiffer polyacrylamide hydrogels generated increase contractile stress.[151] Yet another

study found that differentiated cardiomyocytes most resembled adult cardiomyocytes with respect to contractile activity, myofibril alignment, electrophysiology, direction of calcium flow, organization of mitochondria, and presence of transverse tubules when the cells were maintained on polyacrylamide hydrogels of stiffness similar to that found in myocardial tissue and micropatterned to have a 7:1 length-to-width ratio.[152] Finally, iPSC-CMs maintained on an easy-to-prepare Matrigel mattress had rod-shaped morphologies and increased sarcomere length, contractility, and expression of maturation markers.[153]

Other methods of inducing maturity include electrical stimulation,[154,155] treatment with pharmacological agents such as phenylephrine and angiotensin II,[156] and overexpression of certain genes or microRNAs.[157–159] For example, heterologous overexpression of miR-499 and miR-1 enhanced the differentiation of iPSC-CMs and facilitated electrophysiological maturation of the cells,[157,158] and overexpression of Kir2.1 also promoted electrophysiological maturation.[159]

Some of the shortcomings of iPSC-CMs in the dish can be overcome by fabricating the cells into engineered 3-dimensional structures.[155,160–165] In addition to displaying characteristics that suggest a greater degree of maturity and of similarity to adult cardiomyocytes, iPSC-CMs within these engineered myocardial structures allow for more authentic disease modeling by permitting the analysis of characteristics that are difficult to assess in a dish, including cytoskeletal architecture, force generation, and arrhythmogenesis. In one example, individual iPSC-CMs were seeded on micropatterned fibronectin rectangles designed to mimic the length-to-width ratio of adult cardiomyocytes, providing the investigators with a better way to assess sarcomere disorganization in *TAZ*-mutant iPSC-CMs.[50] The same investigators used a “heart-on-chip” platform in which iPSC-CMs were seeded onto thin elastomers micropatterned with fibronectin lines and supported by glass coverslips, thus fabricating muscular thin film tissue constructs in which the impaired contractility of *TAZ*-mutant iPSC-CMs could be observed and precisely measured.[50] In another study, 3-dimensional tissues were generated by combining iPSC-CMs with human mesenchymal stem cells along with collagen and fibrinogen, which were then embedded within 3-dimensional micropatterned matrices that incorporated cantilevers to directly measure forces generated by the tissues.[42] This platform demonstrated that tissues made from *TTN*-mutant iPSC-CMs had decreased contractile function and were impaired in their response to β-adrenergic stimulation.

Although we have focused on iPSC-CMs in this section, the same limitations and considerations—lack of consistency, lack of purity, lack of maturity, and lack of ability to fully model disease in the dish—apply to all in vitro differentiated cell types. For example, the deficiencies of iPSC-hepatocytes with respect to metabolic functions are well documented.[166] A large cohort of iPSC-hepatocytes that were subjected to whole-genome gene expression profiling were found to be quite distinct from both primary human hepatocytes and whole-liver samples; the iPSC-hepatocytes faithfully modeled some hepatocyte-specific eQTLs but failed to appropriately model other eQTLs.[107] Just as with iPSC-CMs, the past decade has seen substantial effort being devoted to overcoming these obstacles for each particular differentiated cell type.

Need for Biological Replicates

The vagaries of iPSC generation, single-cell clonal expansion, propagation of cells in culture for numerous passages, imperfect differentiation protocols, and even stochastic variation between environment conditions in side-by-side wells on the same dish can all introduce confounders into even the most careful, rigorous iPSC-based study designs. This makes it mandatory to use biological replicates—ideally, many biological replicates—when using iPSCs for disease modeling.

We have already discussed in Study Design how the use of unmatched iPSC lines from different individuals makes a study susceptible to confounders, and how the best remedy is to use a large number of cell lines in each experimental group. Yet even if the cell lines being compared are isogenic cell lines generated by genome editing, it is still problematic to draw conclusions from a comparison of single lines representing each experimental group (eg, a single wild-type line versus a single knockout line) for several reasons. First, genome-editing tools carry the risk of off-target effects such as cleavage and mutagenesis (indels) at sites in the genome other than the desired target site. Off-target effects present a potential confounding factor that could account for any difference observed between single cell lines. Although initial studies with genome-editing tools (especially CRISPR-Cas9) in transformed cultured cell lines suggested that they might incur a high frequency of off-target effects, subsequent studies using whole-genome sequencing in hESC and iPSC clones targeted with genome-editing tools indicated that for any given clone, the number of off-target mutations was very few, perhaps zero.[34–36] However, these same studies brought to light a second issue of arguably greater concern, which is the possibility that the individual clones could carry a large number (dozens to hundreds) of unique single-nucleotide variants. Such variants are not expected to result from the use of genome-editing tools (which preferentially produce indels), which suggests that single-nucleotide variants arise spontaneously in culture in different cells at different times. This means that any 2 clonal lines expanded from single cells within the same stock will almost certainly differ with respect to some variants and thus are not truly isogenic, which raises the possibility of a confounding factor. The third issue arises from the practice of expanding clonal lines from single cells, a process that inevitably exerts selection pressure on the cells and thereby alters the cells in unpredictable ways—if not at the genetic level, then quite possibly at the epigenetic level. The resulting differences between cell lines, whether with respect to proliferation rate, capacity for differentiation into the desired cell type, or some other factors, can also act as confounders.

With all of these potential confounders in play, the best way to ensure that any observed phenotypic differences between experimental groups are authentic is the use of multiple clonal cell lines per experimental group. If one uses 2 cell lines per experimental group, it becomes unlikely that the same off-target effects, single-nucleotide variants, or selective pressures will manifest in both cell lines within a single group and not in any of the cell lines in the other group(s); this becomes extremely unlikely if one uses ≥3 cell lines per group.

In some cases, it might not be feasible to generate multiple cell lines per experimental group. For example, knocking in a variant into 1 or both alleles of an iPSC line via HDR-based genome editing, to generate heterozygous or homozygous cells, might occur with such low efficiency that one would only obtain a single clone with the desired genotype despite

screening hundreds of clones.[107] Even generating that single clone might entail multiple steps: knocking in the variant on one allele, single-cell expansion of the heterozygous clone, knocking in the variant on the other allele, and single-cell expansion of the homozygous clone. With so many manipulations of the cells and passages in culture, one should be greatly concerned about confounding factors. In such cases, one way to ensure that any observed phenotypic differences are genuinely attributable to the genetic variant is to attempt to rescue the phenotype with a complementary genetic alteration, such as by using genome editing to restore the original genotype, or taking an alternative approach (eg, if an introduced variant is thought to result in loss of function of the target gene, to heterologously express the wild-type gene).[103,167] If the phenotypic differences are successfully reversed, then they can be confidently attributed to the genetic variant.

A final consideration is variability within a single clonal iPSC line. Even when grown in the same dish, cells in different wells in the dish can experience subtly different conditions that result in discernible phenotypic effects. These differences can be particularly accentuated with the process of in vitro differentiation, which, as described previously, can vary significantly in efficiency from sample to sample and result in inconsistently heterogeneous cellular mixtures (eg, undifferentiated cells with differentiated cells, or less mature cells with more mature cells).[107,168] With these confounding factors in play, one should generally avoid making comparisons between single samples of cell lines. Rather, it is best to use multiple samples for each clonal cell line, which can be undertaken by simply growing and differentiating the samples in multiple wells in the same dish or in multiple dishes in the same incubator and then processing the samples in parallel. In principle, the greater the concern for inconsistency in growth and differentiation conditions, the greater the number of replicates should be used for each clonal cell line.

iPSCs FOR PRECISION MEDICINE

Use of iPSCs to Perform Patient-Specific Therapeutic Screening

One important goal of precision medicine is to prescribe the right dose of the right drug for the right patient at the right time. A substantial amount of energy has been devoted to pharmacogenomics, defined by the US Food and Drug Administration as the study of variations of DNA and RNA characteristics that are related to drug response. A number of pharmacogenomic applications related to cardiovascular drugs such as warfarin, clopidogrel, and statins are being explored.[169] Such applications rely on genotyping of 1 or a few DNA variants that have been found to be strongly associated with either a beneficial response or an adverse response to the medication in question. Arguably, this is an overly simplistic approach to achieving the goal of pharmacogenomics; as with most clinical traits and diseases, it is likely that a large number of genomic loci or many factors in one’s genetic background could influence the response to the medication. By virtue of having a perfectly matched genetic background, patient-specific iPSCs can provide a model system that integrates all of the genomic loci involved in the response to the medication. Thus, iPSCs offer an opportunity to better test the efficacy and safety of the medication in vitro without exposing a patient to risk. Indeed, it should be possible to assess the effects of several alternative medications in an iPSC-based model and thereby choose the most appropriate

medication for the particular patient, rather than prescribing the medications to patients in general without knowing how each would react and determining the best choice only by trial and error.[170]

However, the utility of iPSC-based models depends on differentiated cells such as iPSC-CMs being faithful proxies for the tissues in the living human being. The investigation of whether iPSC-based models will prove to be sufficiently predictive to warrant their use in clinical decision making is still in its infancy, but initial studies show some promise. In one study, iPSC-CMs generated from patients with LQTS or HCM were observed to be more susceptible to the arrhythmogenic effects of cisapride, a medication that was withdrawn from the US market because of side effects such as ventricular arrhythmias and SCD in patients with conditions such as heart failure and QT prolongation secondary to other medications.[171] In another study, 2 potent blockers of I_{Ks} displayed signs of increased toxicity in iPSC-CMs from a patient with LQTS2 compared with control iPSC-CMs.[172] In yet another study, iPSC-CMs from patients who had demonstrated extreme sotalol-induced QT prolongation exhibited larger increases in field potential durations than iPSC-CMs from patients who showed minimal QT prolongation with sotalol.[173]

In an example that underscores the potential of using iPSC-CMs for personalized treatment, iPSC-CMs were generated from a patient with LQTS and frequent ventricular arrhythmias who had been found to have 2 candidate causal variants, 1 in *KCNH2* and 1 in *SCN5A*. Electrophysiological analysis of the iPSC-CMs revealed sodium channel current defects but no potassium channel current defects, which indicates that the *SCN5A* variant was responsible for the patient's LQTS. The defect was ameliorated with treatment with the sodium channel blocker mexiletine, an effect that was accentuated by increased pacing of the iPSC-CMs but not by addition of a second sodium channel blocker, flecainide.[75] The effects observed in the iPSC-CMs suggested that the optimal treatment for the patient would be to prescribe mexiletine alone and to set a high pacemaker rate (via the patient's implantable cardioverter-defibrillator). Indeed, this was the treatment that had been empirically found to best control the patient's arrhythmias.

Cancer patients who receive the chemotherapeutic agent doxorubicin are at a significant risk for developing cardiomyopathy, particularly with higher doses of the drug, but the mechanistic basis for this drug toxicity is unclear, and there is currently no way to predict which patients will experience this serious adverse consequence upon receiving the drug. iPSC-CMs were generated from 8 breast cancer patients who had received doxorubicin, 4 of whom had developed cardiomyopathy and 4 of whom had not.[174] Upon treatment with doxorubicin in vitro, iPSC-CMs from cardiomyopathy patients had reduced viability compared with those from noncardiomyopathy patients. The former also displayed impaired mitochondrial and metabolic function, impaired calcium handling, decreased antioxidant pathway activity, and increased reactive oxygen species production. Thus, the patient-specific iPSC-CMs were able to recapitulate the vulnerability of the patients to doxorubicin cardiac toxicity, and one could envision using iPSC-CMs to guide the choice of using doxorubicin versus adjusting its dosage to tailor the optimal chemotherapeutic regimen.

In similar fashion, iPSC-CMs from 13 individuals were screened with 21 US Food and Drug Administration–approved tyrosine kinase inhibitors, used to treat various types of cancer, for cardiotoxicity. The results were used to generate a cardiac safety index for the tyrosine kinase inhibitors.[175] Bioinformatics analysis of transcriptomic profiles of iPSC-CMs has been used to predict patient-specific drug-induced cardiotoxicity.[176] Going beyond drug toxicity, iPSC-CMs might also be used to assess a patient's risk of adverse outcomes from other types of environmental exposures; for example, iPSC-CMs have been used as an in vitro model for coxsackievirus B3–induced myocarditis.[177]

Although it might not presently be feasible to routinely generate iPSCs and differentiated cells for every patient, given the effort and expense involved, the examples described above suggest that judicious use of iPSC-based models could be valuable in complex patient cases, particularly when they involve genetic disorders in which the causal mechanism is unclear or when there is a substantial risk that administration of a medication will have grave consequences. Another possible future use of patient-specific iPSCs is to perform drug screening with differentiated cells, to choose among a panel of established medications or even identify novel candidate drugs to treat the patient's condition.

Use of iPSCs to Define Patient-Specific Mechanisms

One of the challenges of studying complex cardiovascular disorders is that different pathophysiological mechanisms can result in similar clinical outcomes. Mutations in different genes or even different mutations in the same gene can have can different consequences at the molecular level but still have the same downstream phenotypic consequences. Patient-specific iPSCs offer the opportunity to dissect the mechanisms directly relevant to the patients' mutations. As related in Use of iPSCs to Perform Patient-Specific Therapeutic Screening, this can be useful for the personalization of therapy, but it can also reap rewards with respect to basic science investigations.

One example of the use of iPSCs to define patientspecific mechanisms is the aforementioned studies on the missense R173W mutation in *TNNT2*.[43,44] Derived from patients with DCM linked to this mutation, iPSC-CMs were found to have epigenetic activation of phosphodiesterase gene expression, a hitherto unrecognized mechanism that plausibly contributes to DCM pathophysiology via the modulation of β-adrenergic signaling. Detailed studies of the iPSC-CMs suggested that the R173W mutant troponin T protein is altered in such a way as to aberrantly localize in the nucleus and interact with the histone demethylase proteins KDM1A and KDM5A, presumably resulting in chromatin remodeling at the genomic sites of the implicated phosphodiesterase genes.

Whether this mechanistic pathway is generalizable to all cases of familial DCM related to *TNNT2* mutations or, even more broadly, to cases of DCM linked to mutations in other genes, remains to be determined. The ability to generate iPSCs from many DCM individuals with many different gene mutations will be invaluable in determining the prevalence of epigenetic activation of phosphodiesterase genes among DCM patients, as well as in elucidating alternative pathogenetic DCM-associated mechanisms. A deeper understanding of all of the potential mechanisms that can result in DCM might lead to a better appreciation of DCM subtypes, with patients being assigned to subtypes based on molecular phenotypes,

whether measured in iPSC-CMs or other proxy tissues. Each DCM subtype might well require its own distinct clinical management strategy in order for patients to be optimally treated, which involves not just choice of medication but other interventions such as lifestyle changes, implantation of devices to prevent ventricular arrhythmias, and pacemaker settings to optimize long-term cardiac function. The same can be said for other phenotypically complex inherited cardiovascular disorders such as ARVC and HCM. Thus, iPSC-based studies could inform precision medicine in ways that go far beyond pharmacogenomics. Indeed, the individualized nature of iPSC-based platforms enables the decoding of patient-specific, cell-autonomous disease development. Evolution from product-focused disease modeling to process-focused disease discovery brings with it the opportunity to understand prephenotypic disease onset and lays the groundwork for targeted individualized therapeutics.[178]

Use of iPSCs to Functionally Annotate Patient-Specific DNA Variants

Variants of uncertain significance (VUSs) are emerging as a significant challenge in clinical genetics, one with enormous implications for precision medicine. With many patients now undergoing exome or genome sequencing, a trend that is sure to increase exponentially in the coming years, some are being found to have novel variants in genes linked to monogenic cardiovascular disorders such as the cardiomyopathies and rhythm disorders described in previous sections. This poses a dilemma to clinical providers as to whether and how to counsel and manage their patients with these findings. Cardiomyopathy and rhythm disorder genes present a special challenge in light of the current recommendation from the American College of Medical Genetics that potentially pathogenic variants in these genes be reported to patients, irrespective of their preference and even if the variants were discovered incidentally from testing related to noncardiovascular conditions.[179] The knowledge can be troublesome, because there is still no reliable means by which to distinguish pathogenic variants from benign variants with respect to gene function. Computational methods to predict pathogenicity have performed poorly in discriminating among established pathogenic and benign mutations,[180] and attempts to use population-based methods to determine pathogenicity (ie, searching public exome databases to determine whether the variants are absent in the general population, which suggests they could be pathogenic) have resulted in patients being incorrectly advised that they have pathogenic mutations in cardiomyopathy genes.[181]

Adding to the complexity of the problem is the fact that variants in the same gene can give rise to very different diseases. For example, *MYH7*, *MYBPC3*, and *TNNT2*, the genes most commonly found to be mutated in familial HCM, are also mutated in substantial proportions of familial DCM cases. Different variants in *SCN5A* have been linked to LQTS3, Brugada syndrome, idiopathic ventricular fibrillation, sick sinus syndrome, and even familial DCM. For such genes, a VUS presents not only the question of whether it is pathogenic or benign but also the question of which disease it might cause in the patient, with serious implications for the management of the patient.

What is needed to fulfill the promise of precision medicine for patients in light of the discovery of VUSs is an efficient and reliable method of functionally annotating the variants.

This would allow clinical providers to properly advise patients as to whether their particular variants put them at risk for disease and, ideally, which disease, as well as to recommend a proper course of action. It would also guide providers in deciding whether to offer to test family members for the same variants. iPSCs could potentially provide a platform for the functional annotation of variants. Unlike biochemical assays or heterologous expression of mutant genes in cultured transformed cells, iPSCs could be differentiated into the relevant cell type (eg, iPSC-CMs for cardiomyopathy gene variants) and more authentically recapitulate the phenotypic consequences of a given variant.

In principle, one could derive an iPSC line from a patient with a VUS, differentiate it into the relevant cell type, and compare its phenotypes with a collection of iPSCs from patients with the disease of concern and from healthy individuals. This might help to determine whether the patient is at risk for the disease based on the patient's genetic background. Although this outcome would certainly be useful, this approach has several shortcomings. First, it will be challenging to generate enough iPSC lines to keep pace with the large number of patients who will be found to have VUSs in cardiovascular disease genes. Second, even if a patient's iPSC line suggests a predisposition for the disease, it does not establish whether the variant in question is a driving factor for the disease and thus needs to be screened in family members. Third, there is the possibility that the patient's iPSC line might turn out to be a poor differentiator with respect to the relevant cell type.

Genome editing enables a more systematic approach to the problem of VUSs. One can envision a standardized platform in which a well-characterized, high-quality (with respect to differentiation), wild-type iPSC line would be used for the insertion of many VUSs. All known variants in a gene (cataloged in clinical databases such as ClinVar) could be compared in a series of isogenic cell lines, thus removing many potential confounders and isolating the effects of the variants. Upon establishing and calibrating the platform with respect to the known variants (ie, being able to classify variants based on their pathogenicity and the disease to which they predispose, if any), novel variants discovered in patients could be introduced into the platform and then appropriately classified. Although it might not be possible to predict with 100% reliability whether a given variant will definitely cause a disease in a given person (given that genetic background and environmental factors will likely be involved), the functional annotation would certainly be more reliable than any existing approaches, such as computational methods.

In the long term, one could envision this kind of platform being integrated into genetics clinics such that any patient who undergoes clinical sequencing and is found to have a known variant in a cardiovascular disease gene could be given a functional interpretation of the variant immediately or, if found to have a novel variant, could be given a functional readout within a few months, which would then be used to inform the patient's prognosis and management.

PROMOTING THE USE OF iPSCs FOR BIOMEDICAL APPLICATIONS

Establishing and Facilitating Access to Biobanks

As described in Methods to Derive New iPSC Lines, the generation of large numbers of iPSC lines is not a trivial undertaking and at present requires a substantial amount of funding. One way in which to amortize the investment of generating cell lines is to make them widely and easily available to the scientific community, so that many investigators can make use of the same cell lines rather than needing to generate cell lines of their own. This will be especially helpful when investigators wish to perform large-scale studies using dozens or even hundreds of cell lines. The establishment of iPSC biobanks is a welcome trend in this direction.

The National Heart, Lung, and Blood Institute has invested $80 million in its 5-year NextGen program,[37] which has resulted in the generation and banking of ≈1500 iPSC lines. The California Institute of Regenerative Medicine has invested a comparably large amount of money to generate and bank 9000 iPSC lines from 3000 people. The New York Stem Cell Foundation seeks to build a biobank of 2500 iPSC lines. A number of smaller biobank collections have been underwritten by various international, federal, and state organizations. Collectively, these efforts represent a promising start toward the goal of making genetically diverse cohorts of iPSC lines available to the scientific community.

An additional important step for funding agencies will be to encourage grant recipients to routinely collect and freeze peripheral blood cells from any patients recruited for their studies, even if the generation of iPSCs is not the primary intent of the studies. As long as the consent forms signed by recruited patients cover the possibility of the generation of iPSC lines from their cells, the frozen samples will be available for iPSC generation indefinitely and could potentially be used by investigators in the future, upon request. Although such collections could and should be maintained by individual local institutions, they could also be maintained on a national scale. The National Institutes of Health’s Precision Medicine Initiative Cohort Program has the goal of recruiting 1 million people nationwide and collecting an extensive amount of medical data, as well as millions of biospecimens that will be stored in a biobank at Mayo Clinic. It is unrealistic to expect that iPSC lines will ever be made from all 1 million participants. However, as genetic and clinical data become available to investigators, it could be feasible for an investigator to select specific individuals with genotypes and phenotypes of interest and request that iPSC lines be generated from those individuals’ stored biospecimens, with measures taken to ensure their anonymity.

Collectively, the emerging concept of stem cell “theranostics” incorporates the triad of disease modeling, cellular diagnostics, and personalized therapeutics.[182] The workflow of disease modeling starts with patient-derived iPSC biobanks to enable molecular profiling and characterization of defining traits of health and disease. Cellular diagnostics then builds on disease-specific biomarkers to resolve the genotype-phenotype relationships that could offer insight into the innate capacity of individuals to be treated. Personalized therapeutics is based on the knowledge of dysfunctional tissues, which provides the rationale to target the underlying mechanism of disease.

Development of Technologies to Scale Up Production and Differentiation of iPSC Lines

One way in which to more rapidly populate large iPSC biobanks and to empower research making use of large cohorts of iPSCs is to foster the development of technologies to produce iPSC lines at a large scale and to differentiate a large number of iPSC lines in parallel. Such efforts are underway and can be expected to greatly accelerate progress in the field.

The generation of iPSC lines can be made more cost-effective and efficient by simply optimizing the manual steps routinely used to reprogram patient cells. In one report, such optimization enabled a single staff member to generate 20 to 40 iPSC lines at a time, making it feasible for a single small laboratory to generate a few hundred iPSC lines in a year.[183] Other efforts have focused instead on automation. One such attempt resulted in the development of a modular, robotic platform that can perform all of the steps in the process of reprogramming cells from skin biopsy samples; a key feature of this platform entailed the high throughput, pooled selection of polyclonal pluripotent cells derived from each patient sample, which resulted in the iPSCs displaying less line-to-line variation (with respect to global gene expression) than iPSCs resulting from single-cell selection and expansion.[184] The platform was also designed to perform automated differentiations, although the purity of iPSC-CMs was typically around 50%. It was estimated that the automated platform could further increase throughput by 10- to 12-fold and decrease reagent costs by 5- to 6-fold compared with the most optimized manual reprogramming strategy reported to date.[183]

A number of automated systems have been used to maintain hPSCs in 2-dimensional culture conditions, either in flasks or in a 96-well format, the latter being particularly useful for drug screens or testing differentiation conditions.[185–187] An important innovation has been the ability to maintain and expand hPSCs in suspension culture, which allows for large-scale growth of the cells in a more cost-effective manner than with flasks and plates.[188,189] Another key step forward has been the development of platforms to differentiate hPSCs into cardiomyocytes with reasonably high yield (surpassing 80% purity) in suspension culture.[190–193] The use of simulated microgravity increased the purity even further.[194] With continued progress, it might soon be possible to produce hundreds of patient-specific iPSC-CM samples for use in single large-scale experiments with little need for manual intervention. Furthermore, many of these innovations can be readily applied to other differentiated cell types relevant to cardiovascular diseases.

Incorporation of iPSCs Into Drug Development Pipelines to Assess for Toxicity

It has been extensively documented that many drug candidates that had appeared to be safe in preclinical cellular and animal models displayed unacceptable toxicities when ultimately used in human beings; one example is increased risk of ventricular arrhythmias, which has been involved in 28% of drug withdrawals from the market in the United States.[195,196] This perhaps speaks to the lack of fidelity of standard preclinical models to important aspects of human physiology, such as cardiac electrophysiology.

Drug candidates are often tested in Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells overexpressing the hERG channel, which contributes the rapidly activating delayed rectifier potassium current (I_{Kr}) and is a primary mediator of

drug-induced QT prolongation and ventricular arrhythmias. These cell lines are popular because they are easy to maintain in culture, are straightforward to use, and lend themselves to high-throughput drug screening; however, these cell lines are quite distinct from human cardiomyocytes and do not recapitulate important aspects of their physiology (hence the need to overexpress the hERG ion channel in the first place). The lack of expression of ion channels other than hERG has resulted in incorrect assessments of drug effects. For example, verapamil acts on both potassium and calcium channels, with countervailing effects that render the drug neutral overall in both human cardiomyocytes and iPSC-CM models.[197] However, in a system in which only the hERG channel is overexpressed, verapamil produces a result that is interpreted as false-positive toxicity.[198] It is also notable that the aforementioned cell lines are aneuploid and thus could be dysregulated in their responses to drugs for that reason.[199]

Although testing of drug candidates in preclinical animal models might appear to make up for the shortcomings of CHO and HEK cultured cell lines, in fact these animals also have significant disadvantages related to major differences in cardiac physiology compared with humans. The mouse heart naturally beats about 9 times faster than the human heart, with correspondingly briefer APDs, and dependent on different ion channels.[200] The I_{Kr} current, which plays an important role in human cardiomyocytes and is involved in drug-induced QT prolongation, has a negligible role in mouse cardiomyocytes. Genes involved in sarcomere structure and function and calcium handling differ in their expression patterns between the species, which can result in major differences in disease pathophysiology. Although larger animals more closely resemble humans with respect to cardiac physiology, they are not as readily bred and maintained as mice, which makes them less suitable for candidate drug assessment. Moreover, even larger animals can differ profoundly from humans in their responses to drugs.[201]

In principle, primary human cardiomyocytes would be the optimal model with which to test for drug toxicities; however, they are difficult to procure and to maintain in culture and do not lend themselves to high-throughput screening. By comparison, iPSC-CMs are advantageous in all of these respects. As discussed in Differentiation Protocols, although iPSC-CMs have characteristics that mark them as immature relative to adult human cardiomyocytes, they nonetheless have many shared properties that make them appropriate for use in preclinical drug testing as an alternative to currently used cellular and animal models. For instance, in contrast to hERG-overexpressing cell lines, iPSC-CMs, which express hERG at similar levels as adult human cardiomyocytes,[171] correctly determined verapamil to be nontoxic with respect to QT prolongation.[197] Alfuzosin, a drug that can cause QT prolongation via its action on sodium channels rather than hERG and thus is scored as nontoxic in hERG-overexpressing cell lines,[202] was correctly found to be toxic in iPSC-CMs.[197]

In 1 study, iPSC-CMs were used to screen 131 different drugs at 6 different concentrations for cardiotoxicity using a 384-well format, which demonstrated the ability to use iPSC-CMs in high-throughput assays.[203] In another study, iPSC-CMs were used to assess 51 previously characterized compounds for their effects on cardiomyocyte contraction and were found to provide a sensitivity of 87% and specificity of 70%.[204] A more recent study by investigators

at the US Food and Drug Administration confirmed the potential utility of iPSC-CMs for the prediction of proarrhythmic risk of drugs.[205] As iPSC-CMs gain broader recognition as an advantageous system for the testing of candidate drugs, they are starting to be used in drug discovery efforts. For example, a liposomal formulation of doxorubicin, an agent with well-known cardiotoxic effects (as related in Use of iPSCs to Perform Patient-Specific Therapeutic Screening), was tested on iPSC-CMs, leading to limitation of delivery of the drug into cardiomyocytes that prevented signs of toxicity.[206] These findings informed the decision to advance the formulation to phase I clinical trials.

A good case can be made for the routine incorporation of iPSC-CMs into drug development pipelines now, if not as a replacement for some of the currently used preclinical models, then at least as an additional, complementary model. A particular advantage to iPSC-CMs is that they reflect the genetic backgrounds of the individuals from whom they were generated. Because responses to drugs can vary widely depending on particular genetic variants, it would be advantageous to use a panel of iPSC lines from a diverse group of individuals who reflect a diversity of genotypes. It would then be feasible to assess for differences in safety and, if relevant, efficacy across genetic subgroups within the target population. A drug that displayed evidence of toxicity with some but not all iPSC lines would likely be disqualified, whereas a drug that displayed evidence of efficacy with some but not all iPSC lines could be further investigated to define which individual patients would benefit from it versus which would not. Thus, incorporating iPSC-CMs early into drug development pipelines could greatly empower precision medicine.

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*Significant.

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*Modest.

†Significant.

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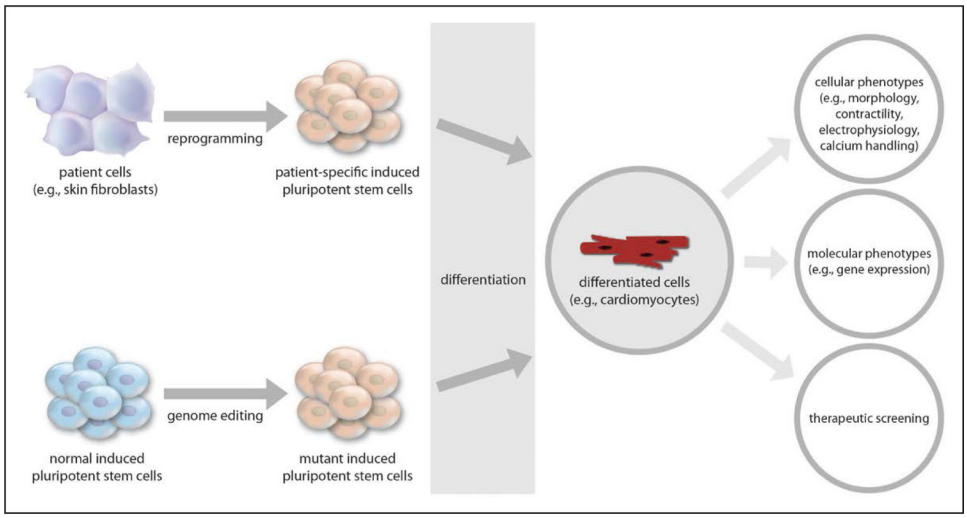

Figure 1.

Induced pluripotent stem cells for cardiovascular disease modeling and precision medicine studies.

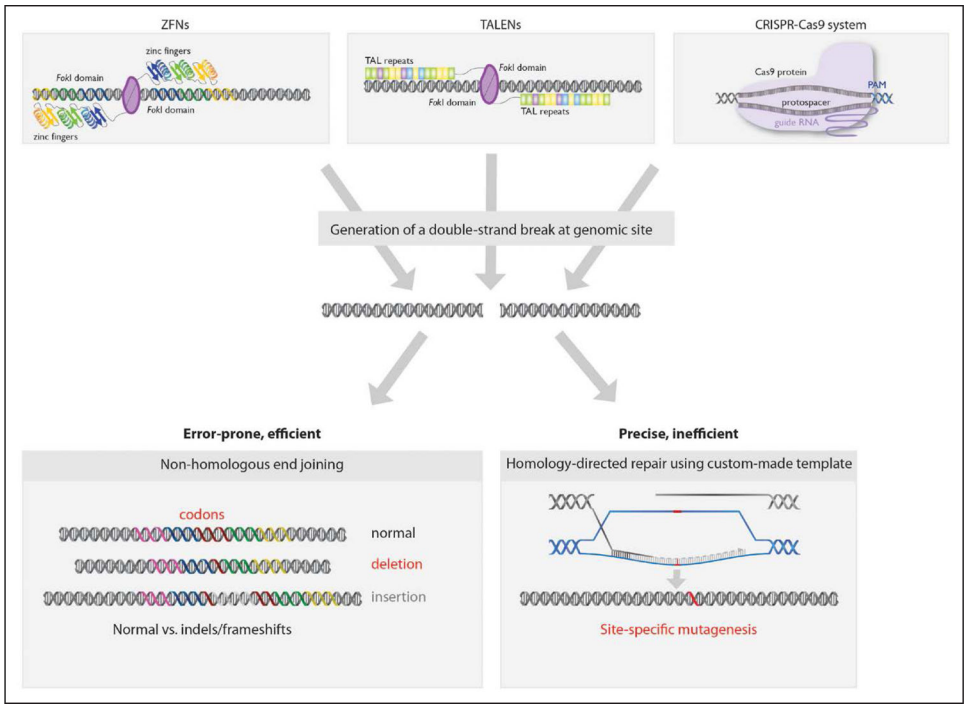

Figure 2. Genome-editing tools for use in induced pluripotent stem cells.

Cas indicates CRISPR-associated; CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif; TAL, transcription activator-like; TALENs, transcription activator-like effector nucleases; and ZFN, zinc-finger nuclease.

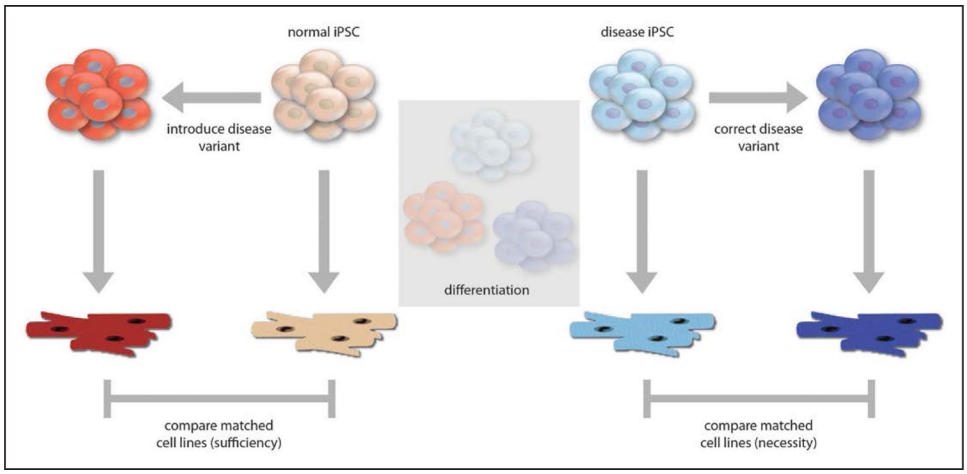

Figure 3. Study designs to assess for sufficiency and necessity of disease variants.
iPSC indicates induced pluripotent stem cell.

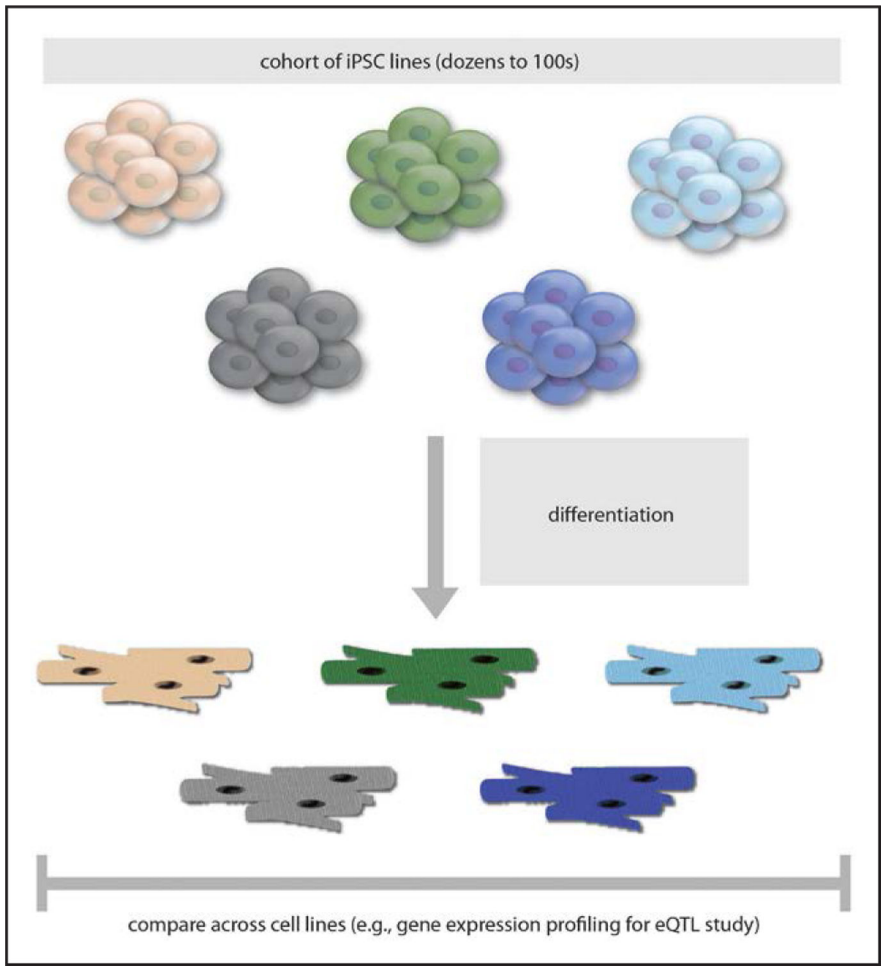

Figure 4. Study design using a cohort of iPSC lines.

eQTL indicates expression quantitative trait loci; and iPSC, induced pluripotent stem cell.

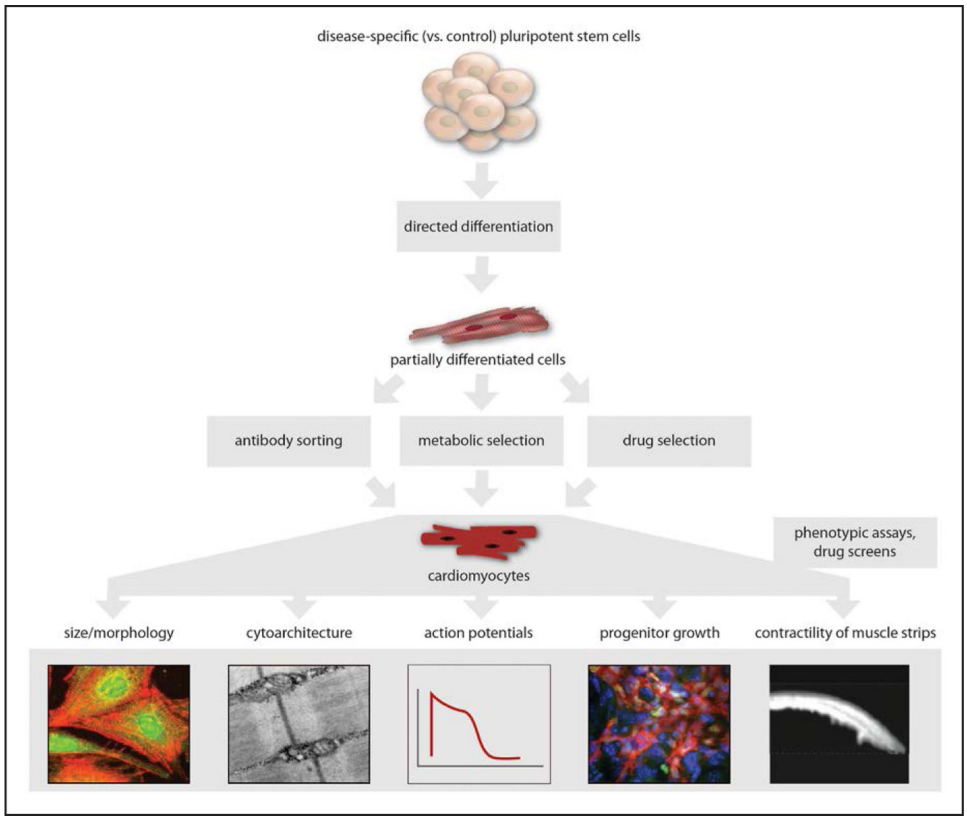

Figure 5.
Cardiomyocyte differentiation.

Table 1.

Differences in iPSC Reprogramming Methods

Feature	RNA Transfection	Sendai Virus	Episomal Vectors	Lentivirus
Efficiency (with fibroblasts)	High	Moderate	Low	High
Reliability (with fibroblasts)	Moderate	High	High	High
Amount of work	High	Low	Low	Very high (requires excision of vector)
Aneuploidy rate	Low	Moderate	High	Moderate
Number of input cells (fibroblasts)	Low	High	High	Moderate
Time to iPSC colony emergence	Low	Moderate	Moderate	Moderate
Efficiency (blood cells)	Not reported	High	High	High
Reliability (blood cells)	Not reported	High	High	High
Special equipment needs	5% O_2 incubator	None	Transfection device and O_2 incubator	None
Proportion of iPSC lines free of reprogramming reagents by passage 5	Very high	None	Moderate	None
Proportion of iPSC lines free of reprogramming reagents by passage 9–11	Very high	High	Moderate	None

iPSC indicates induced pluripotent stem cell.

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Table 2

Partial List of Cardiovascular and Related Diseases Modeled With Human Induced Pluripotent Stem Cells or Human Embryonic Stem Cells

Disease Modeled	Mutated Gene
Dilated cardiomyopathy	*TTN, TNNT2, LMNA, PLN, DES*
Duchenne muscular dystrophy	*DMD*
Barth syndrome	*TAZ*
Hypertrophic cardiomyopathy	*MYH7*
Arrhythmogenic right ventricular dysplasia	*PKP2*
Left ventricular noncompaction	*TBX20, GATA4*
Long-QT syndrome type 1	*KCNQ1*
Jervell and Lange-Nielsen syndrome	*KCNQ1*
Long-QT syndrome type 2	*KCNH2*
Long-QT syndrome type 3	*SCN5A*
Timothy syndrome	*CACNA1C*
Catecholaminergic polymorphic ventricular tachycardia type 1	*RYR2*
Catecholaminergic polymorphic ventricular tachycardia type 2	*CASQ2*
Brugada syndrome	*SCN5A*
Calcific aortic valve	*NOTCH1*
Williams-Beuren syndrome	*ELN*
Familial pulmonary hypertension	*BMPR2*
Familial hypercholesterolemia	*LDLR, PCSK9*
Familial hypobetalipoproteinemia	*PCSK9*
Tangier disease	*ABCA1*
Dyslipidemia	*SORT1*
Maturity-onset diabetes of the young type 2	*GCK*
Insulin resistance	*AKT2*
Hypoinsulinemic hypoglycemia and hemihypertrophy	*AKT2*

Table 3.

Differences Between iPSC-CMs and Adult Human Cardiomyocytes

	iPSC-CMs	Adult Cardiomyocytes
Morphology		
Shape	Round or polygonal	Rod and elongated
Size	20–30 μm	150 μm
Nuclei per cell	Mononucleated	≈25% multinucleated
Multicellular organization	Disorganized	Polarized
Sarcomere appearance	Disorganized	Organized
Sarcomere length	Shorter (≈1.6 μm)	Longer (≈2.2 μm)
Sarcomeric protein – MHC	β>α	β>>α
Sarcomeric protein – titin	N2BA	N2B
Sarcomeric protein – troponin I	ssTnI	cTnI
Sarcomere units – H zones and A bands	Formed after prolonged differentiation	Formed
Sarcomere units – M bands and T tubules	Absent	Present
Distribution of gap junctions	Circumferential	Polarized to intercalated disks
Electrophysiology		
Resting membrane potential	≈ –60 mV	≈ –90 mV
Upstroke velocity	≈50 V/s	≈250 V/s
Amplitude	Small	Large
Spontaneous automaticity	Exhibited	Absent
Hyperpolarization-activated pacemaker (I_f)	Present	Absent
Sodium (I_{Na})	Low	High
Inward rectifier potassium (I_{K1})	Low or absent	High
Transient outward potassium current (I_{to})	Inactivated	Activated
Conduction velocity	Slower (≈0.1 m/s)	Faster (0.3–1.0 m/s)
Calcium handling		
Ca^{2+} transient	Inefficient	Efficient
Amplitudes of Ca^{2+} transient	Small and decrease with pacing	Increase with pacing

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	iPSC-CMs	Adult Cardiomyocytes
Excitation-contraction coupling	Slow	Fast
Contractile force	≈nN range/cell	≈μN range/cell
Ca^{2+}-handling proteins (CASQ2, RyR2, and PLN)	Low or absent	Normal
Force-frequency relationship	Positive	Negative
Mitochondrial bioenergetics		
Mitochondrial number	Low	High
Mitochondrial volume	Low	High
Mitochondrial structure	Irregular distribution, perinuclear	Regular distribution, aligned
Mitochondrial proteins – DRP1 and OPA1	Low	High
Metabolic substrate	Glycolysis (glucose)	Oxidative (fatty acid)
Adrenergic signaling		
Responses to β-adrenergic stimulation	Lack of inotropic reaction	Inotropic reaction
Cardiac α-adrenergic receptor ADRA1A	Absent	Present

cTnI indicates cardiac troponin I; iPSC-CMs, induced pluripotent stem cell–derived cardiomyocytes; MHC, myosin heavy chain; and ssTnI, slow skeletal troponin I.

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