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CONCISE REVIEW

Inherited cardiac diseases, pluripotent stem cells, and genome editing combined—the past, present, and future

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Abstract

Research on mechanisms underlying monogenic cardiac diseases such as primary arrhythmias and cardiomyopathies has until recently been hampered by inherent limitations of heterologous cell systems, where mutant genes are expressed in noncardiac cells, and physiological differences between humans and experimental animals. Human-induced pluripotent stem cells (hiPSCs) have proven to be a game changer by providing new opportunities for studying the disease in the specific cell type affected, namely the cardiomyocyte. hiPSCs are particularly valuable because not only can they be differentiated into unlimited numbers of these cells, but they also genetically match the individual from whom they were derived. The decade following their discovery showed the potential of hiPSCs for advancing our understanding of cardiovascular diseases, with key pathophysiological features of the patient being reflected in their corresponding hiPSC-derived cardiomyocytes (*the past*). Now, recent advances in genome editing for repairing or introducing genetic mutations efficiently have enabled the disease etiology and pathogenesis of a particular genotype to be investigated (*the present*). Finally, we are beginning to witness the promise of hiPSC in personalized therapies for individual patients, as well as their application in identifying genetic variants responsible for or modifying the disease phenotype (*the future*). In this review, we discuss how hiPSCs could contribute to improving the diagnosis, prognosis, and treatment of an individual with a suspected genetic cardiac disease, thereby developing better risk stratification and clinical management strategies for these potentially lethal but treatable disorders.

KEYWORDS

cardiac, CRISPR, differentiation, experimental models, gene targeting, pluripotent stem cells

1 | INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of mortality worldwide. In young people (<40 years), CVD-related deaths often have a genetic origin, with inherited cardiomyopathies and primary

arrhythmia syndromes being the most common.¹ However, even after years of extensive research, treatment options remain quite limited for both of these inherited conditions, with the majority either delaying disease progression but with incomplete efficacy (eg, anti-arrhythmic drugs for treating cardiomyopathies) or causing adverse

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side effects (eg, inappropriate shocks from implantable cardiac defibrillators).^{2,3} One reason for this is that (transgenic) animal models used to investigate the pathophysiology of CVD may differ from humans in cardiac gene expression and physiology, so limiting translational impact.⁴ Because of the finite availability of primary human cardiac tissue, immortalized (cardiac and noncardiac) cell lines expressing a limited repertoire of relevant cardiac genes have been used as alternatives, but these rarely replicate the context and complexity of a functional cardiomyocyte.⁵ Given the shortcomings of these models, a clear need emerged for relevant human-based paradigms to investigate the pathophysiology of cardiac disorders and explore the possibilities for individualized therapeutic strategies.

The discovery that cardiomyocytes could be derived in principle in unlimited numbers from human pluripotent stem cells (hPSCs)—first from human embryonic stem cells (hESCs) and later from human induced pluripotent stem cells (hiPSCs)—opened up the possibility of generating realistic human in vitro models for the heart. Subsequent studies have demonstrated that these hPSC-derived cardiomyocytes (hPSC-CMs) express the required ion channel, signaling, and contractile proteins for functional excitation-contraction coupling,⁴ therefore closely reflecting human cardiac physiology and in many cases responding appropriately to pharmacological compounds.⁶ Combined with the ability to derive hiPSCs from virtually any patient means in vitro models of heart disease can be created in a way that previously was not possible.

In this review, we provide an overview of how hPSCs have been used to model congenital CVD, focusing on the primary arrhythmia syndromes and cardiomyopathies. Such models are providing crucial insights into the pathogenesis of numerous disease-associated mutations. However, these diseases are characterized by large variations in disease phenotypes, in part due to the presence of additional genetic variants that are also of clinical importance both diagnostically and prognostically. We review how recent developments in genome engineering are enabling the contribution of genetic mutations and polymorphisms in cardiac disease to be dissected using hPSCs. Through various examples, we outline how this could improve risk stratification and clinical management of patients with inherited CVD, offering unprecedented opportunities in the field of personalized medicine.

2 | hPSC MODELS OF INHERITED CARDIAC DISEASES

The ability to reprogram somatic cells to a pluripotent state has fundamentally reshaped studies into the genetic basis of human cardiac disorders by facilitating bedside-to-bench research (ie, reverse translational medicine). Here, patients can be selected based on their clinical symptoms and/or specific genetic mutations, and hiPSCs derived that genetically match the patient. This makes hiPSCs excellent models for studying monogenic disorders because the underlying genetic etiology is usually strong enough to induce the pathological phenotype in vitro. Complex cardiac diseases with (putative) polygenic

Significance statement

A key feature of monogenic cardiac diseases is their variable expressivity and incomplete penetrance. Genetics plays a major role in this through both the primary genetic mutation and the presence of additional genetic variants that modify the disease outcome. Genome sequencing of individuals, as well as ongoing genome-wide association studies, identifies many possible variants that could be involved but need to be validated. This study discusses how the combination of human pluripotent stem cells and genome editing offers a potential link in not only investigating the pathogenicity of these primary genetic mutations, but also in determining the contribution of modifying variants. Understanding this should improve clinical decision-making and advance the field toward making precision medicine a reality.

causes can also be modeled using hiPSCs,⁷ but here it is more difficult to identify contributing genetic factors due to the multifaceted complexity of these diseases.

After derivation and characterization, including genotype confirmation, patient-derived hiPSC can be differentiated in vitro into cardiomyocytes as well as other somatic cell types (reviewed in Reference 5). Initial protocols used either serum-containing media or coculture with endodermal cells to generate hiPSC-derived cardiomyocytes (hiPSC-CMs). However, differentiation efficiency was highly variable, making it labor intensive to repeat and scale-up experiments. More recently two-dimensional monolayer protocols have become more favored. Coupled with improvements in culture media, including the replacement of serum, it means it is now possible to generate large numbers of hiPSC-CMs efficiently and to consistently reach purities approaching 90%. However, the resulting hiPSC-CMs generally more resemble fetal rather than adult cardiomyocytes,⁸ which remains an issue for modeling CVDs that manifest postnatally. Despite this, hiPSC-CMs typically reflect at least some of the pathognomonic features observed in the patient and thus have been used to investigate the genotype-phenotype relationship for numerous inherited cardiac disorders, especially primary arrhythmic diseases and cardiomyopathies. In this section, we summarize some of the key findings over the last decade with a list of hPSC models for these disease subtypes published over this period provided in the Supplemental Table S1. More detailed overviews of many of these disease models are provided in other recent reviews.⁹⁻¹⁴

2.1 | Primary arrhythmic diseases

Advances in DNA sequencing are rarely more apparent than in the transformative effect on the clinical diagnosis and management of cardiac arrhythmias. From identifying the first genetic mutation causing an arrhythmia in 1995,^{15,16} there are now more than 28 genes

implicated in inherited cardiac arrhythmic disorders.¹⁷ Most genotyped cases are caused by mutations in cardiac ion channels and are known as cardiac channelopathies. This includes the long QT syndromes (LQTS), short QT syndromes (SQTS), Brugada syndrome (BrS), and catecholaminergic polymorphic ventricular tachycardia (CPVT). These arrhythmic diseases are inherited typically in an autosomal dominant manner, although there are a few subtypes that are autosomal recessive (ie, Jervell and Lange-Nielsen syndrome [JLNS]). Despite the multitude of different genes associated with these disorders, their hallmark feature is an alteration to the characteristic ECG pattern of the heart, either at rest or in response to exercise, fever or pharmacological challenges.¹⁸ Clinically, this can predispose the patient to cardiac arrhythmias, ventricular fibrillation, and sudden cardiac death (SCD). Many different channelopathy subtypes have been modeled using hiPSCs derived from patients (Supplemental Table S1). Typically the resulting hiPSC-CMs have been characterized by patch clamp electrophysiology, multielectrode arrays, or calcium flux assays,¹¹ which has revealed the expected alterations to the action potential (AP), field potential (FP), or Ca²⁺ transients, respectively.

LQTS type 1 (LQT1) was the first ion channelopathy modeled using hiPSC-CMs. In this study, hiPSCs were derived from two LQT1 patients with the heterozygous missense mutation R190Q in the gene *KCNQ1*.¹⁹ The LQT1 hiPSC-CMs reflected the salient features of LQT1 including a reduction in the AP repolarizing current I_{Ks} , resulting in a prolongation of the AP duration (APD) and occurrence of arrhythmic events when exposed to the β -adrenergic agonist isoproterenol. Similar phenotypes have also been observed in additional patient-derived LQT1 hiPSC-CM models (Supplemental Table S1). Homozygous mutations in *KCNQ1* can lead to JLNS, an autosomal recessive disorder characterized by a prominent extension of the QT interval and deafness.¹⁸ hiPSC-CMs derived from a JLNS patient or genetically engineered to have a homozygous mutation in *KCNQ1*, showed the expected pronounced APD prolongation and susceptibility to drug-induced arrhythmias.²⁰

LQT2 has also been extensively modeled using patient-derived hiPSCs (Supplemental Table S1). LQT2 patients carry autosomal dominant mutations in the gene *KCNH2*, which encodes the channel responsible for mediating the other main AP repolarizing potassium current, I_{Kr} . Here too, LQT2 hiPSC-CM models have reproducibly shown an expected increase in AP and/or FP duration, as well as arrhythmogenicity and a smaller I_{Kr} compared with control hiPSC-CMs.^{21,22} Additionally, molecular characterization of the LQT2 hiPSC-CM models has confirmed that many *KCNH2* missense mutations result in a trafficking defect of the ion channel.^{23,24} Recently, Lumacaftor, a drug known to act on channel trafficking and approved for treating cystic fibrosis, was shown to restore trafficking of the ion channel in LQT2 hiPSC-CMs but only for certain mutations.²⁵ Excitingly, the first attempt to validate these findings in two of the patients whose hiPSC-CMs had shown a response, indicated a significant shortening of their QTc interval when they were also treated with the corresponding clinically approved compound.²⁶ These findings emphasize the importance of understanding the complex interplay between an aberrant genotype and molecular phenotype to pave the way for more personalized medicine.

LQT3 is the third most common form of LQTS. It is caused by gain-of-function mutations in the sodium ion channel gene *SCN5A*.¹⁸ hiPSC-CMs from LQT3 patients also showed prolonged APDs due to delays in sodium channel inactivation resulting from a persistent inward sodium current.^{27,28} This phenotype could be rescued upon treating the LQT3 hiPSC-CMs with the clinically approved Na⁺ channel blocker, mexiletine.²⁷ LQT1-3 account for more than 90% of genotyped cases, with the remainder consisting of the officially recognized LQTS subtypes 4-15, as well as LQT16 which is currently awaiting formal approval.¹⁸ The electrophysiological characteristics of LQT7 (otherwise known as Andersen-Tawil syndrome) caused by mutations in *KCNJ2*, and LQT8 (Timothy syndrome) due to mutations in *CACNA1C* have also been modeled in patient-specific hiPSC-CMs.^{29,30} Similarly, hiPSC-CMs with mutations in the calmodulin-encoding genes that cause LQT14 and LQT15 show the expected APD prolongation and disruption of L-type Ca²⁺ channel activity.³¹⁻³³

Like LQTS, mutations in several genes have been associated with BrS with the most common being *SCN5A* loss-of-function mutations. The hiPSC-CMs derived from BrS patients with *SCN5A* mutations have shown the expected reduction in I_{Na} density and upstroke velocity, shorter APD, as well as increased arrhythmic activity.³⁴ Additionally, certain *SCN5A* mutations can result in a combination of both BrS and LQT3 and are referred to as "overlap syndromes." These too have been modeled using patient-derived hiPSCs with the hiPSC-CMs showing the electrophysiological properties of both syndromes.^{35,36} However, hiPSC-CMs from BrS patients without *SCN5A* mutations did not show sodium channel dysfunction or the BrS phenotype.³⁷ As the disease typically occurs in adulthood, the lack of a disease phenotype in the hiPSC-CMs could be due to the immaturity of the hiPSC-CMs or the absence of other nongenetic contributors (ie, fibrosis and environmental factors).

Although less prevalent, SQTS also causes shortening of the QT interval, predisposing patients to atrial and ventricular arrhythmias and SCD.³⁸ To date, six subtypes have been identified with the causative genes also linked to LQTS and BrS. Currently only SQTS1 caused by missense mutations in *KCNH2* has been modeled in hiPSC-CMs, with the models reflecting the main phenotypic features of SQTS1 including shortened repolarization, abnormal Ca²⁺ transients, and arrhythmic activities.³⁹⁻⁴¹ To model more complex electrophysiological phenomena, multicellular sheets of the patient hiPSC-CMs were generated.⁴⁰ The SQTS1 hiPSC-CM sheets revealed accelerated and more stabilized rotor dynamics which might facilitate reentry formation and contribute to the severe arrhythmogenic phenotype seen in SQTS patients.

Another arrhythmogenic disorder successfully modeled in patient-derived hiPSC-CMs is CPVT. The most common forms of CPVT are due to mutations in the intracellular Ca²⁺-regulating genes ryanodine receptor 2 (*RYR2*) or calsequestrin-2 (*CASQ2*), causing CPVT1 and CPVT2, respectively. Dysfunction of either of these key players in cardiac excitation-contraction coupling triggers abnormal intracellular Ca²⁺ handling and signaling, as well as delayed after depolarizations (DADs) that are exacerbated under catecholaminergic stress. Numerous hiPSC-CM CPVT models (Supplemental Table S1) have also exhibited arrhythmogenicity

and dysregulated Ca^{2+} homeostasis, either in the absence or presence of catecholaminergic stress.^{42,43} Recently, a new subtype, CPVT3, was identified in multiple unrelated patients exhibiting overlapping features of LQTS and CPVT.⁴⁴ The patient-specific hiPSC-CMs, which contained mutations in trans-2,3-enoyl-CoA reductase-like protein (*TECRL*) and not in any known arrhythmia-associated genes, reflected the CPVT phenotype with abnormalities in Ca^{2+} handling, prolonged repolarization, and increased frequency of DADs.

2.2 | Cardiomyopathies

A second group of CVDs extensively modeled using hiPSC-CMs are the inherited cardiomyopathies. Numerous mutations in various pathways crucial for cardiac function have been linked to the most common cardiomyopathies—dilated (DCM), hypertrophic (HCM), and arrhythmogenic (ACM); although the possible underlying genetic cause has only been identified in approximately 25% of DCM, and 50% of HCM and ACM cases.⁴⁵ These cardiomyopathies are predominantly characterized by disorganized sarcomeres, potentially resulting in reduced myocardial function and heart failure.⁴⁶ However, mutation carriers exhibit substantial variability in disease severity, with some lifetime asymptomatic while cardiac dysfunction triggers SCD in others. Therefore, understanding the pathogenesis at the subcellular level (ie, the sarcomere) could facilitate the development of treatments aimed at preventing disease progression. This can only be achieved with hiPSC-CMs due to other model systems typically not reflecting the clinical phenotype observed in patients.

Despite having very heterogeneous genetic causes, DCM is clinically characterized by ventricular dilation and impaired systolic contraction, which can lead to ventricular arrhythmias.⁴⁶ To date, hiPSC-CMs have been used to investigate the disease phenotype caused by mutations in multiple genes including sarcomeric and nuclear proteins, as well as Ca^{2+} homeostasis regulators (Supplemental Table S1). Modeling DCM using hiPSC-CMs has been met with some success, with these *in vitro* models exhibiting aspects of the disease phenotype including deficiencies in sarcomeric organization, Ca^{2+} handling, and contractile force.^{47,48}

HCM is one of the most common genetic cardiac diseases affecting ~1:500 individuals and is typically caused by genetic mutations in sarcomeric components or sarcomeric-associated proteins. A clinical phenotype of HCM is thickening of the left ventricular wall in the absence of other factors (eg, hypertension and valve disease).⁴⁹ Although the majority of people with familial HCM are asymptomatic, extensive and severe myocyte disarray has been observed in autopsied patients following SCD.⁴⁹ Here too, key features of the disease have been reproduced in hiPSC-CMs derived from patients with mutations in sarcomeric proteins as well as in kinases (Supplemental Table S1). These include increased cell size, sarcomeric disarray, and more frequent arrhythmic events such as DADs.^{50,51} Some studies have also observed features such as increased *MYH7* gene expression and nuclear accumulation of the transcription factor NFAT,^{50,52} although whether these are disease specific remain contentious.¹³ Finally,

dysfunctional Ca^{2+} dynamics appears to be a key pathological mechanism observed in hiPSC-CM models of HCM, although this is not consistently reflected in changes to contractile force or kinetics.^{53,54}

Finally, although ACM was initially believed to be a disease affecting just the right ventricle and was characterized by ventricular arrhythmias and fibrofatty tissue deposits in the myocardium,⁵⁵ the identification of its genetic basis as well as the phenotyping of large patient cohorts determined that both ventricles could be affected.⁵⁶ The predominantly hereditary disease affects ~1:5000 individuals and is associated with genetic mutations mainly in desmosomal proteins.⁵⁵ As a consequence of late disease onset and the involvement of epicardial cells in mediating the fibrofatty tissue infiltration,⁵⁷ investigating the ACM phenotype and pathophysiology in hiPSC-CM models has been challenging. However, by metabolically maturing the hiPSC-CMs, increased lipogenesis and apoptosis plus abnormal Ca^{2+} handling were detected in the cardiomyocytes with *PKP2* mutations.^{58,59} Also, electrophysiological dysfunction was observed in hiPSC-CMs with a mutation in *DSG2*.⁶⁰

3 | IMPROVING hiPSC CVD MODELS THROUGH GENETIC ENGINEERING

As the previous section illustrates, the past decade has seen an incredible number of hiPSC lines derived from CVD patients where the disease-causing mutation is known or suspected. Although these lines have subsequently been used to successfully model the disease *in vitro*, the question frequently arising is what the most appropriate control is to confidently define and identify the disease phenotype. Until recently, most studies established the pathogenicity of these disease-associated mutations by comparing one or two patient-derived hiPSC lines with one or two hiPSC lines derived from apparently “healthy” control individuals (Supplemental Table S1 and Figure 1). The issue here is that not only might the disease-associated mutation contribute to the observed differences between the lines, but additional confounding factors could also influence the disease penetrance (Figure 2).

In particular, differences in genetic background are a major potential confounder. Some studies have attempted to mitigate this by comparing the patient hiPSC-CMs to those generated from related individuals without the suspected pathogenic mutation or disease phenotype^{27,50} (see Supplemental Table S1 for complete list). However, even if the control is derived from a first degree relative, only ~50% of the genome is shared. Furthermore, many nongenetic factors can influence the behavior and phenotype of the hiPSC-CMs, including different reprogramming methodologies, the epigenetic profiles of the hiPSC lines, and potential to differentiate into the desired cell type.⁶¹

To reduce these effects, most hiPSC cardiac disease modeling studies have focused on patients with highly penetrant monogenic diseases and a clear autosomal dominant inheritance pattern, meaning the severity of the disease phenotype will generally outweigh any noise caused by confounding factors from the control line. But this does not demonstrate that the phenotype is directly or solely caused by the suspected mutation(s). Similarly, it is difficult to detect subtle

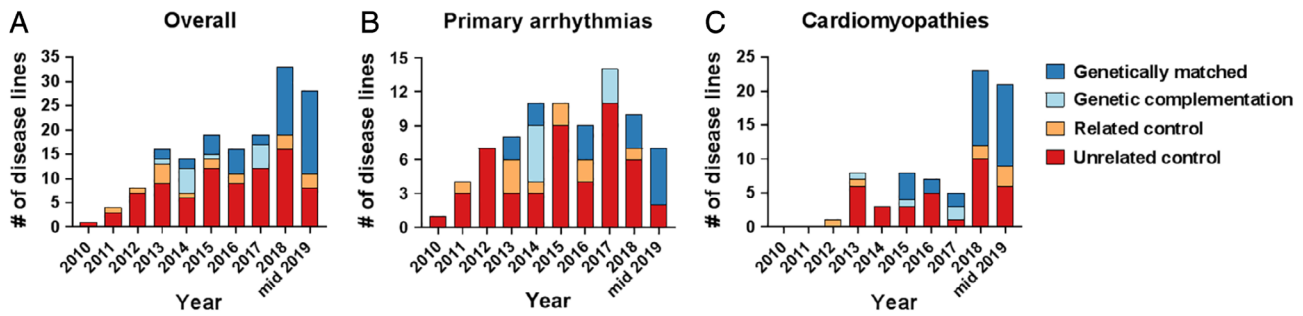


FIGURE 1 Classification of human pluripotent stem cell-derived cardiomyocyte (hPSC-CM) disease models based on the type of control the model was compared with and grouped by year of publication. A, An overview of both primary arrhythmic and cardiomyopathy disease models combined; B, C, show each of these inherited cardiac diseases, respectively. In instances where multiple control types per disease model were reported, classification was based on the predominant control type used to detect the disease phenotype. The list of disease models used in the analysis is provided in Supplemental Table S1. This list was generated by initially examining referenced articles from multiple comprehensive reviews published in the field,⁹⁻¹⁴ with more recent articles identified by performing a PubMed literature search using the query: [“pluripotent stem cell”[Title/Abstract] AND ((arrhythmia[Title/Abstract] OR channelopathy[Title/Abstract]) OR cardiomyopathy[Title/Abstract])] AND (“2018”[PDAT]: “3000”[PDAT])]. The resulting publication list was then screened for additional relevant publications that were then examined manually. When the same hPSC-CM disease model was referred to in more than one publication, only the original reference that described either the isolation of the patient material or the generation of the line was included, unless a different type of control was used in the subsequent publication

phenotypic differences in a clinical syndrome that are due to distinct genetic variants using this approach. Some of these issues can be addressed by comparing large numbers of disease and control hiPSCs (ie, >4 lines per group) under standardized conditions,⁶² thereby improving the resolution for detecting disease-specific cellular phenotypes. The growing number of hiPSC biobanks containing disease lines from multiple individuals, as well as ethnically diverse cohorts of control hiPSC lines will make such studies possible.⁶³⁻⁶⁵ However, it is still labor intensive to generate hiPSC-CMs from multiple lines and subtle phenotypes will remain difficult to resolve.

Alternatively, genetic complementation studies can be used to directly test the genotype-phenotype relationship. For example, this can be achieved with viruses, RNA interference or transgenic approaches to either overexpress or repress the gene or specific variant implicated as being disease causative. Indeed these approaches have all been used to examine the pathogenicity of both channelopathy- and cardiomyopathy-causing cardiac variants⁶⁶⁻⁶⁹ (see Supplemental Table S1 for complete list). Although the genetically modified hiPSC-CMs exhibited the expected phenotypic changes, as the introduced constructs are heterologously expressed, the resulting lines might not accurately reflect the molecular mechanisms underlying the disease pathology.

Fortunately, developments in the hiPSC field have coincided with advances in gene targeting technology. Through homologous recombination, paired “disease” and “control” hPSCs can be generated that in principle differ only by the putative disease-causing mutation, thereby eliminating confounding factors arising from hPSC derivation and genetic background. If the variant is expected to cause a subtle phenotypic difference, then the use of genetically matched disease and control lines is even more critical. Indeed the potential of this for CVD modeling was first demonstrated by correcting a *KCNH2* mutation identified in a LQT2 patient, and reversing the electrophysiological phenotype in the resulting hiPSC-CMs.²³ Introducing the mutation into a wild-type hESC led to the hESC-CMs displaying the disease

characteristics, confirming the pathogenicity of this mutation. Here, conventional gene targeting procedures similar to those originally established for mouse ESCs were used,^{70,71} but this was time-consuming, inefficient, and resulted in DNA fragments related to enrichment strategy remaining in the locus following the modification.

The development of endonuclease-based gene editing systems (eg, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR-Cas9) have made it significantly easier to correct or introduce genetic defects in hiPSCs. These systems introduce double-strand breaks (DSBs) at specific sites in the genome that are then repaired endogenously by the cell—most frequently by error-prone nonhomologous end joining (NHEJ). However, if a DNA template with homology to the sequence surrounding the DSB is present, this can also be achieved by homology-directed repair, often without the need for a selectable marker to be included in the template. Although the principles and unique attributes of each of these gene-editing systems are described in greater detail elsewhere,⁷² a critical aspect is that the DSB dramatically increases the frequency of homology-mediated genomic changes occurring compared with conventional gene targeting methods.⁷³ This efficiency and ease-of-use, especially with the CRISPR-Cas9 system compared with other methods, means it is now feasible to generate genetically matched hiPSC lines within ~2-6 months,⁷⁴ thereby allowing any phenotypic differences to be more reliably attributed to the identified genetic variant. Indeed, the percentage of publications using genetically matched controls has doubled from ~20% in 2015 to ~40% in 2018 (Figure 1). Interestingly, the proportion of studies using genetically matched controls is higher when modeling cardiomyopathies (~50%) than for arrhythmic diseases (~30%), possibly due to the broader variability in disease expressivity and penetrance observed in these contractile diseases.

Although CRISPR-Cas9 has clearly revolutionized the disease modeling field, this genome editing tool is not without challenges. One of these is the need for a protospacer adjacent motif (PAM) sequence for the Cas9 nuclease to anchor to. This can be an issue when trying to

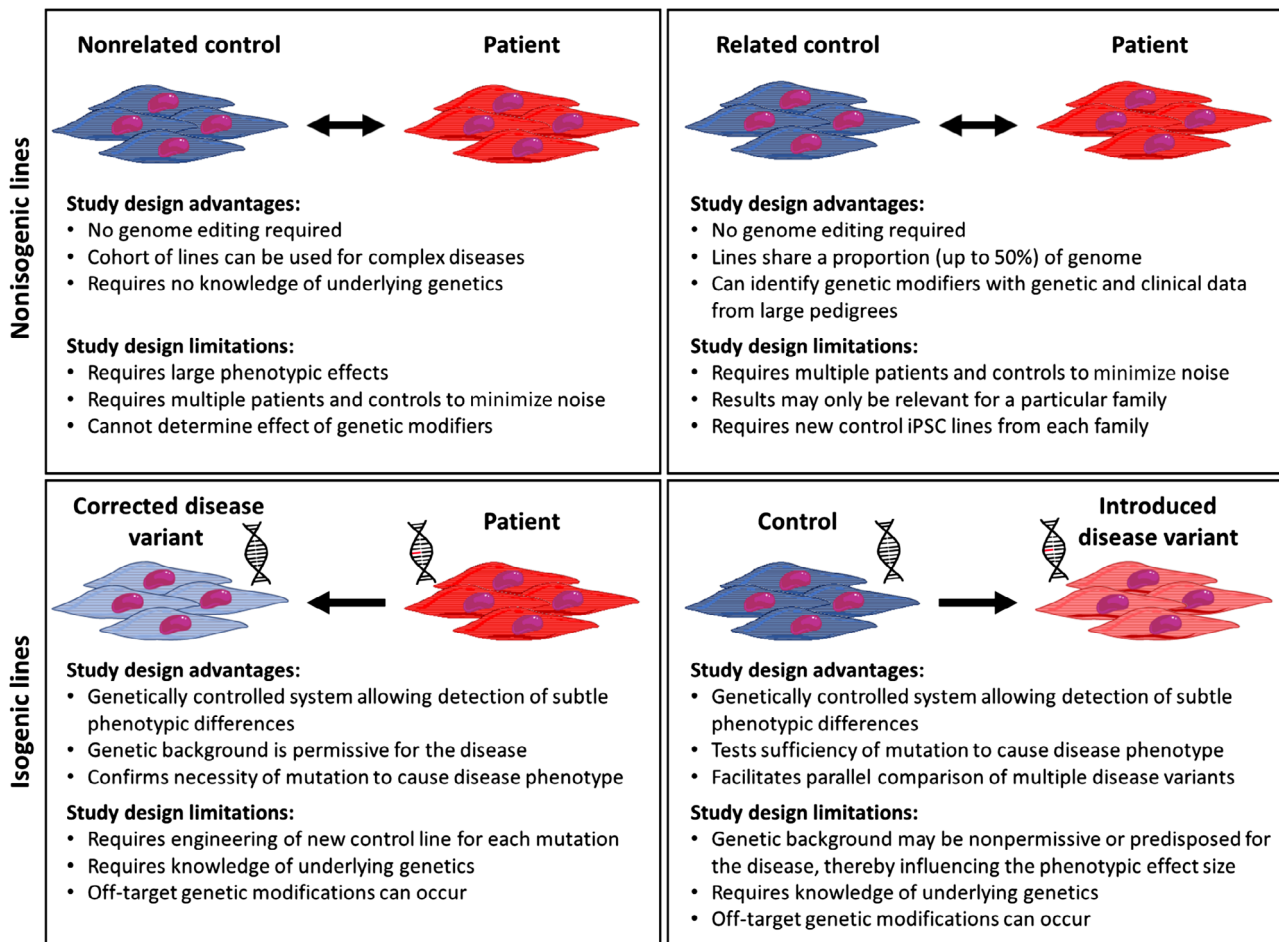


FIGURE 2 Comparison of strategies to model cardiac diseases using human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs). The disease phenotype can be evaluated in the patient-derived human-induced pluripotent stem cell (hiPSC)-CMs using nonisogenic controls (either genetically unrelated or related). Alternatively, to evaluate the pathogenicity of a genetic mutation, the variant can be either introduced into a control hPSC line or corrected in a patient-derived hiPSC line. The genetically matched pairs of “disease” and “control” hPSCs can then be differentiated to cardiomyocytes and the resulting genotype-phenotype relationship investigated

introduce or correct at a specific genetic location if such a motif is not within the same vicinity, although this has been addressed to some extent by the development of alternative Cas9 variants with varied PAM requirements.⁷⁵ Perhaps a greater impediment is inefficient on-target editing, with substantial variability even occurring between target sequences in proximity (ie, 10-20 nucleotides) to each other. These differences might be due to the local chromatin structure, with editing efficiencies reduced if the target sequence is bound by nucleosomes.⁷⁶ In some instances, CRISPR-Cas9 can also prove to be too efficient with biallelic editing occurring at the target site, in which one allele is modified as intended but an insertion or deletion occurs in the other allele due to NHEJ repair.⁷⁷ However, overall editing efficiencies are typically between 2% and 10%,^{78,79} and if the desired genetic modification is not identified after screening ~200 colonies, it is often better to redesign the targeting approach.

Additionally, genome editing approaches are dependent on the identification of a candidate disease-linked variant and are thus best suited for studying monogenic diseases. If the genetic cause is unknown, but the disease appears to be monogenic based on family

inheritance patterns, then deriving hiPSC lines from both affected and unaffected family members might be a better approach to determine the underlying pathogenic mechanism. Similarly, it is presently too labor-intensive to use gene editing to study polygenic diseases as these are due to multiple variants that individually have small contributing effects to the disease phenotype. Here, using large numbers of unrelated patient and control hiPSC lines for which extensive clinical data is also available is likely to be more informative.

The generation of genetically matched hPSC lines by either introducing or correcting the disease-associated mutation fulfill different purposes (Figure 2). Introducing a suspected pathogenic variant into a control hPSC line and observing the expected disease phenotype confirms the variant's pathogenicity, and indicates that it is likely to be sufficient to cause the disease in the patient in whom it was originally identified. If the mutation is pathogenic, then this approach will usually be quicker and cheaper than deriving an hiPSC line from the patient and subsequently correcting the variant. Furthermore, the putative mutation can be introduced into a control hiPSC line that has already been extensively characterized and validated, not only genetically (ie, genome

sequenced), but also in terms of differentiation efficiency and functionality of the resulting hiPSC-CMs. This study design also facilitates the side-by-side comparison of multiple disease variants in a single genetic background. However, if no disease phenotype is observed, this does not necessarily mean that the variant is benign. If the mutation is not fully penetrant and the control hiPSC line chosen carries protective genetic modifiers, then the disease phenotype might be obscured. In this instance, correcting the mutation in the patient-derived hiPSCs demonstrates the necessity of that variant in contributing to the disease phenotype. As the patient exhibits the disease, the patient must carry the combination of modifying alleles required for disease manifestation.

When investigating the pathogenicity of a particular variant, genetically matched comparisons should be performed in multiple hiPSC clones, or more ideally, in at least two unrelated cell lines to evaluate the reproducibility of the disease phenotype. This is because endonuclease-based editing systems carry the risk of also inducing unintended mutations at off-target sites in the genome,^{80,81} although this does not appear to occur frequently in hiPSCs.⁸²⁻⁸⁴ However, a greater potential concern are the single nucleotide variants that individual clones can acquire spontaneously during culture and from the selection pressure associated with clonal isolation steps.^{85,86} This means that genetically edited pairs of hiPSCs are not truly isogenic and these acquired variants could act as (new/irrelevant) confounding factors.

Although currently less than five variants are typically characterized per study, we anticipate there will be increased interest in establishing panels of hiPSC lines containing either several different mutations within a single gene or in multiple genes. Such collections will be immensely valuable in precision medicine initiatives and their applications are discussed in the next section. Indeed, platforms are starting to emerge that allow such CVD panels to be developed rapidly and without requiring an individualized targeting strategy for each variant.⁸⁷ This study demonstrated the possibility of simultaneously generating genetically matched hiPSC lines containing *TNNT2* mutations associated with DCM in a scalable manner. Although only *TNNT2* cDNA could be introduced which would prevent intronic single nucleotide polymorphisms (SNPs) such as those associated with increased risk of heart failure⁸⁸ being investigated, further modifications to this approach will likely enable larger DNA fragments to be exchanged, thereby overcoming such issues.

4 | FUTURE PERSPECTIVES

With developments in hiPSC technology coinciding with similar progress in genome sequencing and high-throughput pharmacological screening, the door has opened to use hiPSCs in precision medicine strategies. The expectation is that hiPSC-CMs can predict the response of the patient from whom they were derived to certain drugs. While still in early stages, recent studies provide optimism that this approach will be useful both for inherited as well as acquired cardiac disorders.^{27,89-92} However, when these technologies are also combined with recent advances in genome editing, potential uses for

hiPSCs in precision medicine go beyond solely pharmacogenomic screens. Rather, it offers the opportunity to determine how a patient's genotype leads to a disease phenotype, and a chance to dissect the mechanisms underlying this. In this section, we discuss some of these applications and their potential value for basic science as well as in the clinic.

4.1 | Classifying variants of uncertain significance

Genetic screening of large panels of genes is now a cost-effective clinical tool for establishing a molecular diagnosis in individuals with a suspected cardiomyopathy or arrhythmia disorder. Not only can identifying the causal mutation support the clinical diagnosis, but it may also offer prognostic and therapeutic value for the patient, and indicate the usefulness of cascade screening in other family members for presymptomatic treatment.⁹³ However, such testing can identify hundreds of nonsynonymous coding variants in an individual. Distinguishing which are pathogenic from benign remains a significant challenge, in particular when it is classified as a variant of uncertain significance (VUS).⁹⁴

The potential for using hiPSCs in combination with CRISPR/Cas9 gene editing to decipher the pathogenicity of such variants was recently demonstrated. In the first example, hiPSCs were derived from a patient diagnosed with LQTS and confirmed to carry a novel VUS in *KCNH2*.⁹⁵ Upon correcting the VUS, the electrophysiological abnormalities observed in the patient hiPSC-CMs were rescued, while introducing the homozygous variant into a control hiPSC line confirmed its pathogenicity. In a separate study by the same group, an hiPSC line was generated from an asymptomatic individual carrying a HCM-associated genetic variant in *MYL3* but predicted in silico to be "likely pathogenic."⁹⁶ However, by correcting the variant in the patient-derived hiPSCs as well as introducing the variant homozygously, they were able to demonstrate that in fact the VUS was benign.

Although these two vignettes demonstrate how to incorporate hiPSCs into a pipeline to clinically evaluate a patient's VUS, there are issues with such an approach. Mainly it is practically impossible and too time-consuming to generate hiPSC lines from all patients carrying a VUS in a cardiac disease-linked gene, genetically correct the variant, and evaluate the pathogenicity. Therefore, alternative strategies utilizing already established and functionally characterized control hiPSC lines are also being investigated. In one case, a *KCNJ2* VUS identified by whole-exome sequencing in a LQT7 patient was overexpressed in commercially available hiPSC-CMs.⁹⁷ The transiently transfected hiPSC-CMs mirrored the phenotype observed in the patient, supporting causality of the variant. However, as already discussed, overexpression approaches may not completely reflect the situation in the adult heart where loss of function of an ion channel may be compensated through altered expression of other channels.⁹⁸ To minimize this issue, genome editing was used to introduce a *CACNA1C* VUS identified in a LQT8 patient into a hiPSC line previously established from a healthy volunteer.⁷⁴ A prolonged APD was observed in the gene-edited hiPSC-CMs, leading to the variant being reclassified "likely pathogenic."

It is also possible to combine this approach with integrase-based gene editing methods,⁹⁹ avoiding inefficiencies associated with CRISPR/Cas9 methods for introducing mutations and meaning a VUS identified in genetic testing can be functionally interpreted within a few months. Being able to evaluate a VUS so rapidly could result in such a screen being incorporated into the patient's treatment strategy. As a proof of concept, this approach was used to evaluate a *TNNT2* VUS identified in a patient with severe HCM between the patient's clinical appointments.⁸⁷ However, in this case, the VUS did not appear to be pathogenic, and the causal mutation remained unidentified. This also illustrates a limitation of evaluating a VUS using a wild-type hiPSC line. A negative result does not necessarily prove that the VUS is benign as the cell line may lack a permissive genetic background for detecting the disease phenotype. Therefore, it is important that lines used for such evaluations are also well characterized at a genetic level. In instances where the VUS appears to be benign, additional donor hiPSC lines with different genetic backgrounds or a modified hiPSC line predisposed to the disease could be also used to confirm the classification. Regardless of these issues, such a screening platform is still likely to be more informative than existing computational and population-based methods.

4.2 | Modeling clinical heterogeneity observed in patients

Clinical management of patients diagnosed with primary arrhythmic and cardiomyopathy diseases is also complicated by the variable disease phenotypes observed among mutation carriers. Indeed it has been suggested that, as for many Mendelian disorders, variability in disease severity and incomplete penetrance are "more rule than exception."¹⁰⁰ Although factors such as gender, age, medication, and exercise are known contributors to this clinical heterogeneity,¹⁰¹ the genotype of the mutation carrier also plays a major role. For example, compound mutations (more than one primary disease-causing mutation) are estimated to be present in ~10% of patients with LQTS,¹⁰² which can account for their greater risk of cardiac events compared with family members with single mutations. Similarly, the type and location of a nonsynonymous mutation within a gene can also explain differences in disease severity observed within some disease subtypes.^{103,104} Finally, additional modulatory genetic factors are also suspected to play a role.¹⁰⁵ A key question is whether hiPSCs can be used to identify these genetic modifiers, as well as understand the molecular and cellular consequences of compound mutations or different nonsynonymous mutations in the same gene (Figure 3). If hiPSC models are sufficiently sensitive to detect these genotype-phenotype correlations, this could lead to improved risk stratification and clinical decision-making for patients.

An early study speculated that this might be possible with the disease phenotype less pronounced in hiPSC-CMs derived from an asymptomatic mutation carrier compared with a related symptomatic patient.²² However, as no lines were genetically matched, these differences could also be attributed to clonal variability from the

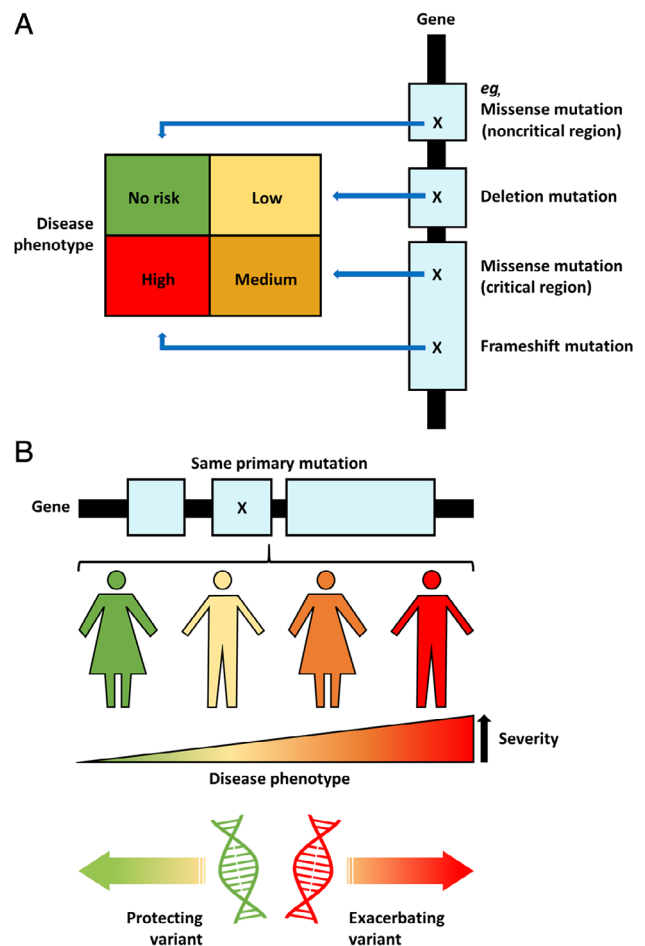


FIGURE 3 Contributing genetic factors to the variable disease expressivity and penetrance observed in patients with monogenic cardiac diseases. **A**, Variability in the disease risk observed between patients classified with a particular disease subtype can be due to the location (noncritical or critical region of the encoded protein) and type (ie, synonymous, deletion, missense, and frameshift) of primary mutation within a gene. **B**, Variability in disease severity even among individuals with the same primary mutation can be due to additional genetic variants that can either protect from or exacerbate the disease symptoms

reprogramming. Another study from the same group underscored the need to study such primary genetic mutations in the same genetic background.¹⁰⁶ Isogenic pairs of hiPSC-CMs were generated from three family members by either correcting or introducing a mutation in alpha-cardiac actin (*ACTC1*) associated with HCM. Although arrhythmogenesis was evident in all hiPSC-CMs with the *ACTC1* mutation, considerable variation in contractile abnormalities was observed between the cell lines including the absence of some contractile defects in the hiPSC-CMs generated from the line derived from the healthy brother in which the mutation had been introduced. These findings highlight how single rare variants can be the central cause of the disease, but also how the (epi)genetic background of the patient, such as through the presence of additional genetic variants or methylation modifications to sarcomeric proteins,¹⁰⁷ can influence the pathogenic phenotype observed.

Recent work also demonstrated the possibility of using hiPSC-CMs to study compound mutations.¹⁰⁸ Genetic sequencing of a multi-generational DCM family identified novel variants in the sarcomeric gene tropomyosin (*TPM1*) and the costameric gene vinculin (*VCL*), with all DCM family members carrying both mutations. Through a combination of gene editing of hESCs and derivation of hiPSCs from control and DCM patients, it was demonstrated that each variant individually reduced contractility in hiPSC-CMs. Although they used an unrelated line, the greatest impact appeared to be in hiPSC-CMs containing both variants, supporting the hypothesis that the variants act synergistically to perturb cardiomyocyte contractility and sarcomeric organization and that both genetic insults are required to cause DCM in the patients.

Finally, the potential to use hiPSC models to identify previously undiscovered genetic modifiers was illustrated in a study of a large family in which several members carried a pathogenic mutation in *KCNH2* but showed variable expressivity of LQT2.¹⁰⁹ Electrophysiological analysis of hiPSC-CMs from severely affected family members displayed prolonged APs compared with hiPSC-CMs from mildly affected first-degree relatives, mirroring the clinical genotype-phenotype discordance and suggesting that there were genetic factors exacerbating and/or providing protective effects. Combining these electrophysiological results with sequencing data revealed two new disease modifying gene variants that could explain the disease variability observed in the family. Further functional vetting demonstrated that the variant in the *KCNK17* channel was protective, while the *REM2* variant was aggravating.

4.3 | Validating genetic associations using hiPSCs

A key factor in the above studies being able to identify either compound mutations or modifying risk alleles was the availability of both genetic and clinical information from related individuals collected over multiple generations.^{108,109} Although such data are now more frequently available, any findings made may only be relevant to that family cohort, and often such clear genotype-phenotype concordance will not be apparent. Genome-wide association studies (GWAS) enable the discovery of potential candidate modifying genes and variants from larger unrelated patient populations. For example, GWAS of QT interval duration have identified SNPs in a number of candidate genes, including *KCNQ1* and *NOS1AP*.¹¹⁰⁻¹¹² Subsequent studies in smaller, heterogeneous populations of LQTS patients have demonstrated that these risk alleles are also associated with an increased risk of cardiac events in LQTS patients.^{113,114} By combining hiPSC models of LQTS with genome sequencing and gene editing technologies, there is now the opportunity to validate these variants as genetic modifiers and investigate their mechanism of action. Such studies will demonstrate the possibility of using hiPSCs in a systems genetics approach to develop a multi-locus genetic risk score that could assist in identifying LQTS patients that could benefit from more vigilant monitoring for arrhythmic events. Biobanks of hiPSCs could provide large enough cohorts to both test and discover novel associations between SNPs

and cardiomyocyte phenotypes, while genome editing techniques would allow more targeted approaches for both a mechanistic understanding as well as identification of the risk variant. Although such an approach has not been demonstrated yet using hiPSC-CMs, there have been examples in the study of metabolic alterations in hiPSC-derived hepatocyte-like cells,^{115,116} as well as in combining GWAS data with gene editing to identify noncoding risk variants contributing to the pathogenesis of Parkinson's disease or multiple vascular diseases.^{117,118}

5 | CONCLUSIONS AND CHALLENGES

As the above sections illustrate, hiPSCs are proving invaluable for disease modeling and evaluating candidate variants that may underlie the genotype-phenotype discordance observed in patients with inherited cardiac diseases. However, it is not without its challenges. Those related to genome editing approaches have been discussed, but limitations at a cellular and functional level also exist. These include the perennially raised issues regarding the immaturity and subtype specificity of the hiPSC-CMs, as well as variability both in differentiation efficiency and functionality of the resulting hiPSC-CMs. These concerns have been comprehensively discussed elsewhere,^{8,119-122} and there are substantial efforts focused in solving them. For hiPSC-CM maturation, various approaches have been used including physical, chemical, or electrical stimulation, as well as coculture of hiPSC-CMs with other relevant cell types—all of which have shown varying levels of improvement. Similarly, refinements to differentiation and enrichment strategies have led to highly pure populations of subtype-specific hiPSC-CMs now being produced. Finally, efforts to standardize processes and experimental conditions have also reduced variability in downstream functional readouts.^{123,124} The challenge now is to develop high-throughput platforms to address the phenotyping bottleneck that is occurring due to the surfeit of disease lines being generated. Advances in automated methods to simultaneously measure electrophysiology, calcium transients, and contractility parameters¹²⁵ will be of great benefit to the field.

Although this review has focused on the disease phenotype found in cardiomyocytes, some of these genetic cardiac diseases, such as BrS and ACM, also affect other cell types present in the heart and thus contribute to the overall pathological phenotype observed in the patient.¹²⁶ Recent developments in the generation of in vitro hiPSC-derived 3D cardiac tissues that incorporate multiple cardiac cell types (ie, vascular, smooth-muscle, and epicardial cells) are powerful tools for studying the phenotypic effects caused by the interactions between these different cells.¹²⁷⁻¹²⁹ Although such heterotypic cell models are still in the early stages of development, 3D engineered cardiac tissues are being used to investigate contractile dysfunction caused by cardiomyopathies with promising results.^{47,54,130,131}

Despite these challenges, hiPSC technology has made significant contributions to cardiovascular research and drug development over the last decade. The ability to generate hiPSC-CMs from any patient, combined with their genetic and clinical information, has led to

successes in unraveling the disease mechanisms specific for that mutation and/or patient, as well as refining treatment strategies for that individual based on these discoveries.^{26,27} Combining this with genetic engineering and genome sequencing developments, we now have unmatched opportunities to accelerate the efficiency and resolution by which we can evaluate variants suspected of having a pathological role and examine the impact GWAS-identified polymorphisms have on the disease phenotype. Such advances offer the possibility of delivering patients from the “genetic purgatory” that the discovery of a VUS can cause,¹³² as well as improve patient-specific risk stratification strategies. This lays the groundwork for tailoring clinical management to that of the individual and gives a glimpse of the future that precision medicine offers.

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CONFLICT OF INTEREST

The authors indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

L.B.: conception and design, literature review, manuscript writing, and figure design; C.G.: literature review; C.M.: final approval of manuscript; R.D.: conception and design, manuscript writing and figure design, financial support, and final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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