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Advances in arrhythmogenic cardiomyopathy modeling using human-induced pluripotent stem cell-based models

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ABSTRACT

Arrhythmogenic cardiomyopathy (ACM) is a hereditary and life-threatening cardiac disease that primarily affects young individuals and athletes. Given that ACM is difficult to distinguish from other cardiac disorders, it is challenging to diagnose, and the first clinical manifestation is often sudden cardiac death. Pathophysiologically, ACM is characterized by cardiomyocyte loss, fibrofatty replacement, contractile and electrical dysfunction, and inflammation. Although significant progress has been made in identifying the genetic underpinnings of ACM, the molecular mechanisms driving ACM development and progression remain poorly understood, limiting therapeutic strategies to symptom management and arrhythmia prevention rather than disease modification. To address this gap, advanced ACM models that accurately recapitulate human (patho)physiology are urgently needed. In this review, we examine the current landscape of 2-dimensional and 3-dimensional human-induced pluripotent stem cell (hiPSC)-derived ACM models, highlighting their ability to replicate key pathologic features and uncover disease mechanisms. We discuss emerging insights from hiPSC-based platforms, their contributions to understanding ACM pathophysiology, and the challenges that remain in modeling this complex disease. Finally, we outline future directions for advancing hiPSC-based ACM research, emphasizing the need for more physiologically relevant models to facilitate mechanistic discoveries and therapeutic development.

KEYWORDS Arrhythmogenic cardiomyopathy; Hereditary cardiovascular disease; hiPSC-derived models; Modeling advances; Molecular mechanisms

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Arrhythmogenic cardiomyopathy

Cardiomyopathies are a heterogeneous group of diseases characterized by mechanical or electrical dysfunction of the heart. These conditions may be either hereditary or acquired, and they often lead to premature death or progressive heart failure.[1]

Arrhythmogenic cardiomyopathy (ACM, OMIM #107970) is a hereditary condition that exhibits incomplete penetrance and variable expressivity. Initially classified as a congenital disease affecting the right ventricle, ACM is now recognized as a progressive disorder that primarily affects the right ventricle but can also predominantly involve the left ventricle or affect both ventricles.[2–4] The hallmark of ACM is the replacement of the heart muscle with fibrofatty tissue, which progresses gradually and spreads from the epicardium to the endocardium.[2,5] The prevalence of ACM ranges from 1 in 1000 to 1 in 5000 individuals, and the disease is associated with a high incidence of sudden cardiac death (SCD), particularly in young athletes.[5] In some patients, SCD may even be the first manifestation of the disease.[5,6] Variable expressivity, age of onset, and different manifestations between the 2 sexes may lead to a higher proportion of undiagnosed individuals.[5] In addition, 30%–50% of the cases are exacerbated by nongenetic factors such as inflammation and physical exercise.[6,7]

The onset of ACM typically manifests after childhood, most commonly between the second and fourth decades of

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life. Early symptoms may include arrhythmias, sometimes without noticeable structural changes in the heart. As the disease progresses, pathologic processes such as myocyte death, inflammation, fibrosis, and the accumulation of fibro-fatty tissue occur (Figure 1). In its later stages, ACM leads to heart failure, initially affecting a single ventricle before progressing to biventricular failure.[6,8]

ACM genetics

ACM is typically transmitted in an autosomal dominant pattern, although some recessive forms have been identified presenting as syndromes.[9,10] In certain patients, compound heterozygosity—characterized by 2 variants within the same gene—or digenic inheritance involving variants in different genes may be observed.[11]

The genetic etiology of ACM is established for more than 60% of the cases. Among affected patients, the most frequently mutated genes encode desmosomal proteins, such as plakophilin-2 (*PKP2*, 53%),[12] desmoplakin (*DSP*, 14%),[13] desmoglein-2 (*DSG2*, 14%),[14] and desmocollin-2 (*DSC2*, 5%).[15] Pathogenic variants in the plakoglobin gene (junction plakoglobin [*JUP*], <1%) have also been identified in a smaller number of cases.[11,16]

In addition, several nondesmosomal genes have also been implicated in ACM accounting, altogether, for the remaining 14% of cases.[11] These include those encoding transcription growth factor β3,[17] desmin,[18] transmembrane protein 43,[19] phospholamban,[20] titin,[21] lamin A/C,[22] alpha-T-catenin,[23] obscurin (*OBSCN*),[24] filamin C (*FLNC*),[25] RNA-binding motif protein 20,[26] sodium voltage-gated channel alpha subunit 5 (*SCN5A*),[27] alpha actinin 2,[28] and N-cadherin (*CDH2*).[29]

ACM models

Animal models have played a pivotal role in advancing our understanding of ACM by helping to uncover the role of the pathogenic variants in the development of the disease. Murine models, in particular, have become essential tools for studying genetic cardiac diseases, thanks to advanced genetic manipulation techniques.[30] Early mouse models proved crucial in elucidating the pivotal role of desmosomes in ACM. Through knockout models, researchers were able to describe the role of key desmosomal proteins involved in the disease, whereas knockin and transgenic mice revealed critical insights into alterations in desmosomal length, number, and structure and how these alterations correlate with defects in electrical coupling.[31] However, the murine heart displays substantial physiological differences compared with the human heart, which diminishes its potential as a model for investigating human cardiovascular disease. Notably, the mouse heart exhibits minimal adipogenesis, a hallmark of ACM, which constrains our capacity to fully elucidate the underlying mechanisms of the disease.[32] In addition, differences in ion channel function and calcium handling between mice and humans may influence the results of cardiotoxicity tests, thereby compromising the reliability of using only ACM mice in the context of drug development.[30,33]

Abbreviations

ACM: arrhythmogenic cardiomyopathy

DSC2: desmocollin-2

DSG2: desmoglein-2

DSP: desmoplakin

ECM: extracellular matrix

EHT: engineered heart tissue

hiPSC: human-induced pluripotent stem cell

JUP: junction plakoglobin

MT: microtissue

PKP2: plakophilin-2

To overcome these limitations, in vitro models to study ACM have been explored: primary cardiomyocytes (CMs) from patients can recapitulate the genetic aspects and pathologic features of the disease. However, maintaining primary CMs in vitro is challenging owing to their low proliferative capacity, limited lifespan, and phenotypic dedifferentiation in cell culture.[30] As an alternative, immortalized cell lines such as HL-1 and neonatal rat ventricular CMs have been used.[34] However, both models have important limitations, being HL-1 cells an atrial cell line, not ideal to study the ventricular prevalence of ACM, and neonatal rat ventricular CMs being immature cells.[30]

The usage of human-induced pluripotent stem cells (hiPSCs) offers a powerful alternative to study ACM. By reprogramming somatic cells from patients into pluripotent cells, as first demonstrated by Takahashi and Yamanaka,[35] researchers can generate various types of cardiac cells for disease modeling, such as hiPSC-CMs, hiPSC-endothelial cells (hiPSC-ECs), and hiPSC-cardiac fibroblasts (hiPSC-CFs).[36,37] In addition, the development of prime editing techniques has led to the generation of isogenic controls in which only the pathogenic variant is corrected.[38] These approaches provide a powerful tool to study cells that carry the genetic background of the patient. These hiPSC-derived cells can then be used to create both 2-dimensional (2D) models and 3-dimensional (3D) models for studying ACM. Although 2D monoculture provides a controllable environment with good accessibility to readouts, cocultures and 3D models offer a closer representation of the in vivo environment. One significant advantage of these models is their ability to promote the maturation of hiPSC-CMs, which exhibit a fetal-like phenotype in 2D monocultures.[30,37] This leads to a better modeling of adult cardiac function and maturation of the hiPSC-CMs in terms of calcium handling, sarcomere organization, and metabolic activity.[30] Despite these advancements, current 3D models also face limitations, such as the absence of vascularization, physiological mechanical stimuli, innervation, and immune cells such as monocytes, which play crucial roles in inflammation and tissue remodeling.[30,39] These challenges underscore the need for further innovations to enhance the physiological relevance of these models. A summary of the key milestones that have shaped the development of ACM hiPSC-CM models is presented in Figure 2.

This review summarizes the potential and limitations of hiPSC-derived models in both 2D and 3D systems as tools for studying ACM. We also seek to provide an overview of the models developed to date and to identify those best suited to address specific research applications. Ultimately, we conclude by discussing the current challenges and future

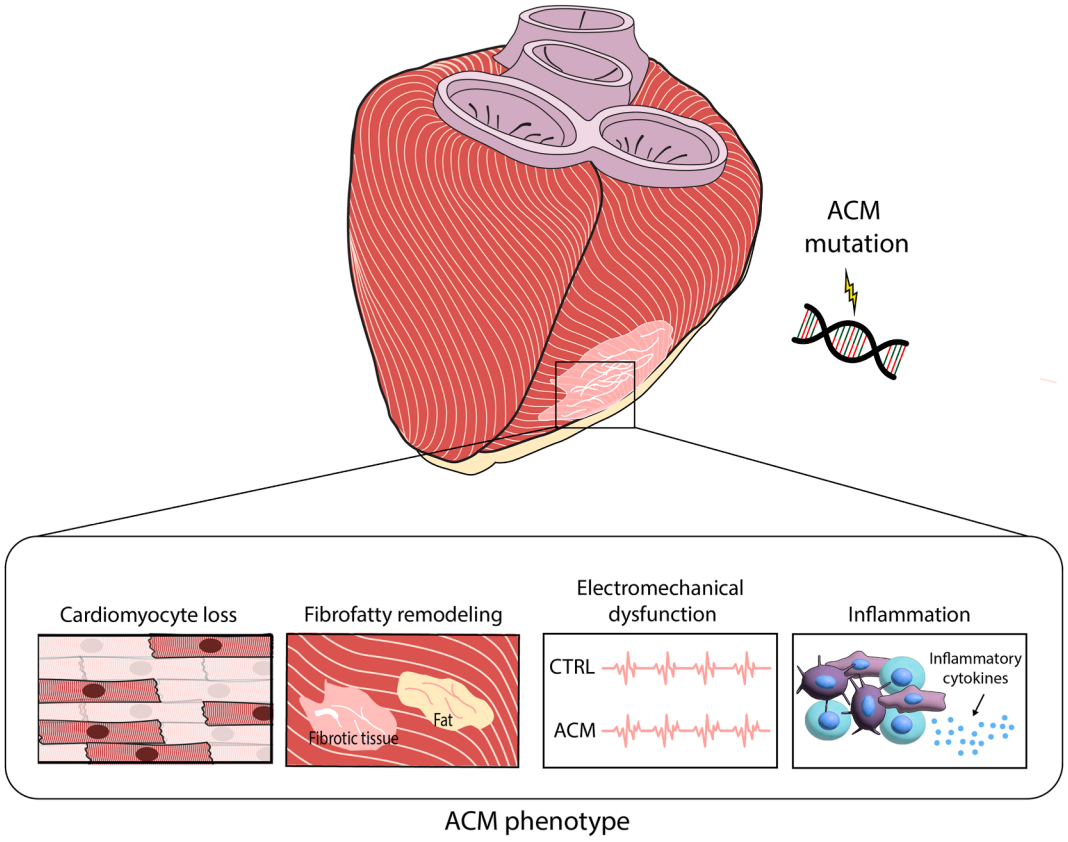

Figure 1
Pathophysiology and cellular hallmarks of ACM. The heart is shown with the right ventricular free wall highlighted, indicating areas commonly affected in ACM. Zoomed-in panels demonstrate the pathologic features at the tissue and cellular levels such as cardiomyocyte loss, fibrofatty remodeling, electromechanical dysfunction, and inflammation. ACM = arrhythmogenic cardiomyopathy; CTRL = control.

perspectives in the field, highlighting opportunities for innovation and advancements in hiPSC-derived models for ACM research.

Traditional 2D hiPSC-based models in ACM

hiPSCs have revolutionized the study of ACM by offering a patient-specific and scalable platform for disease modeling. By recapitulating key features of ACM, including electrophysiological abnormalities, contractile dysfunction, and desmosomal disruptions, these models provide an opportunity to investigate the mechanisms underlying ACM and to examine the molecular consequences of mutations within a patient-specific context. Besides hiPSC-CMs, other cardiac cell types are hypothesized to play an important role in the pathophysiology of ACM. For instance, hiPSC-derived epicardial cells

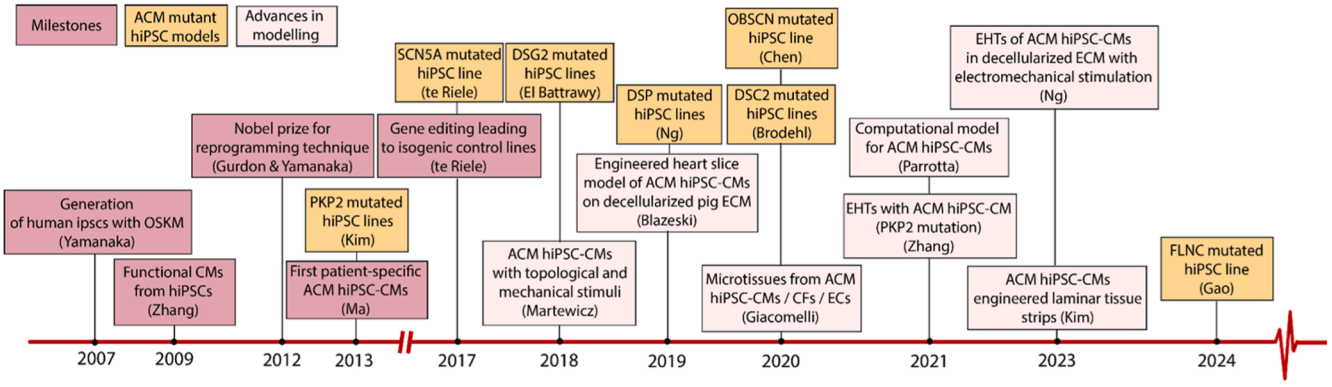

Figure 2
Timeline of milestones in ACM hiPSC-CM model development. Color coding distinguishes general milestones (*pink*), ACM-specific hiPSC-CM models (*yellow*), and advances in modeling techniques (*gray*). ACM = arrhythmogenic cardiomyopathy; CF = cardiac fibroblast; CM = cardiomyocyte; DSG2 = desmoglein-2; DSP = desmoplakin; EC = endothelial cell; ECM = extracellular matrix; EHT = engineered heart tissue; FLNC = filamin C; hiPSC = human-induced pluripotent stem cell; OBSCN = obscurin; OSKM = Oct4, Sox2, Klf4, and c-Myc; PKP2 = plakophilin-2; SCN5A = sodium voltage-gated channel alpha subunit 5.

(hiPSC-EPIs) initiate the processes involved in epicardial-to-fibrofatty transformation that are necessary in ACM progression.[40] In addition, hiPSC-CFs are responsible for extracellular matrix (ECM) remodeling and fibrosis in ACM.[41] In this section, we will highlight the contribution of these 3 different types of cells in the pathogenesis and in the study of ACM.

HiPSC-CMs

HiPSC-CMs are the most studied cell model in ACM research given the profound alterations observed in CMs affected by the disease. The desmosomal changes lead to altered connections in the CMs, which are unable to transmit the electrical signal properly, leading to arrhythmias and cardiac death.[5]

Although numerous hiPSC-CM lines carrying mutations in desmosomal genes (*PKP2, DSG2, DSP, DSC2*) have been developed,[42–45] the past decade has also seen the generation of hiPSC-CM lines with pathogenic variants in non-desmosomal genes (*FLNC, OBSCN, SCN5A)* driven by new insights into ACM genetics.[24,27,46]

Conventional 2D models, where cardiac cells are cultured on flat, rigid substrates such as plastic or glass, have long been a cornerstone of in vitro cardiac research owing to their simplicity, precise control of experimental conditions, and ability to study cellular behavior, functionality, and drug responses (Figure 3A). However, their rigid and static nature does not replicate the myocardium where there is a dynamic interplay between mechanical forces and ECM architecture.[30] Recent advancements have sought to enhance the physiological relevance of 2D models while preserving their experimental accessibility, bridging the gap between traditional 2D cultures and more complex in vivo-like systems.

To introduce mechanical cues reflective of cardiac tissue, Martewicz et al[47] developed deformable substrates with topographic features to mechanically stimulate hiPSC-CMs with a *PKP2* mutation (c.2484C>T) (Figure 3B). Their findings revealed a distinct, disease-specific response to mechanical stress in these hiPSC-CMs compared with healthy cells, underscoring the altered mechanotransduction pathways in ACM.[47] Another innovative method involves engineered

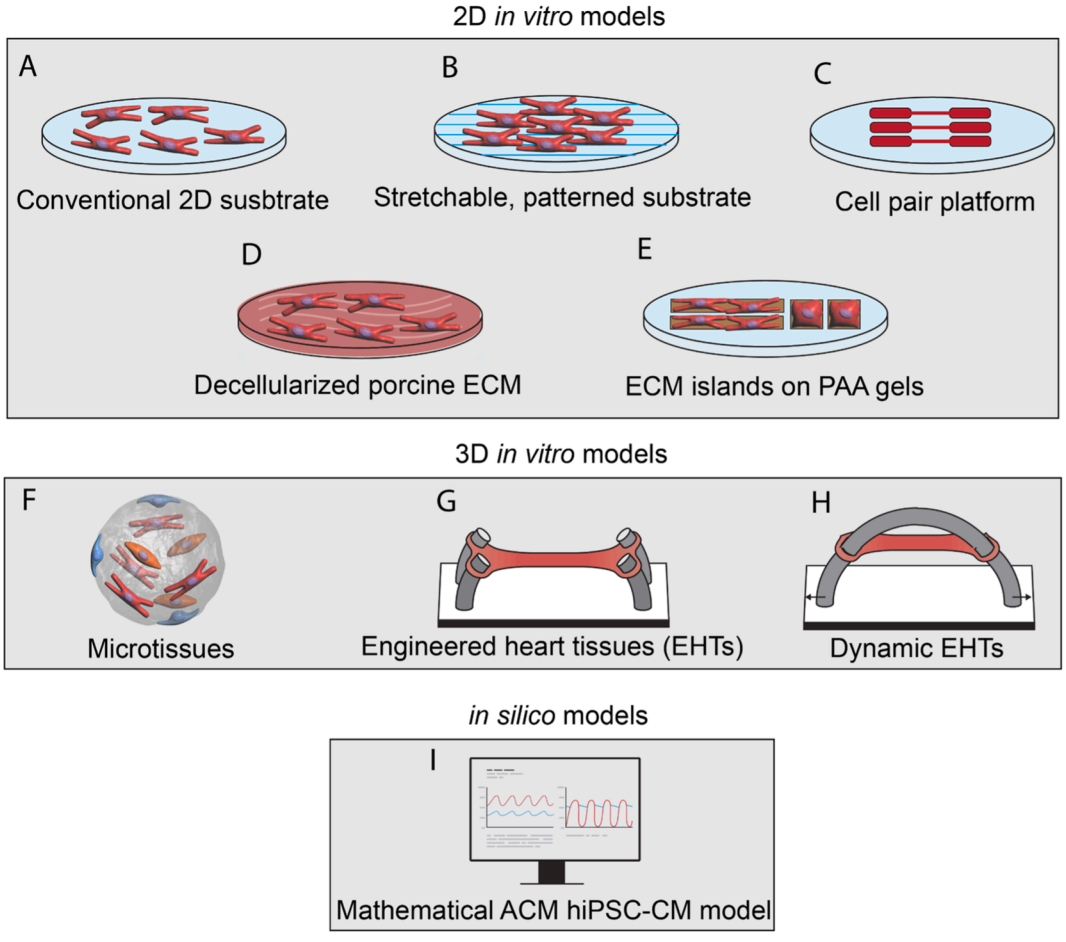

Figure 3
Representative examples of 2D and 3D in vitro models to mimic ACM (patho)physiology. **A:** Conventional 2D culture substrate. **B:** 2D stretchable patterned substrate to mimic the cardiac functional syncytium. **C:** Bioengineered cell pair platform to study the assembly of cell-cell junctions. **D:** Engineered heart slice model consisting of decellularized porcine ECM. **E:** Patterned ECM islands on PAA gels containing fluorescence beads for traction force microscopy. **F:** 3D multicellular microtissue model. **G:** Engineered heart tissue for simultaneous mechanical loading and force contraction measurements. **H:** Dynamic engineered heart tissue. **I:** Computational mathematical model of hiPSC-CMs. 2D = 2-dimensional; 3D = 3-dimensional; ACM = arrhythmogenic cardiomyopathy; CM = cardiomyocyte; ECM = extracellular matrix; EHT = engineered heart tissue; hiPSC = human-induced pluripotent stem cell; PAA = polyacrylamide.

laminar tissue strips, which replicate aspects of native cardiac tissue organization (Figure 3C). Using this approach, Kim et al[48] demonstrated that hiPSC-CMs with a *PKP2* mutation (p.R413) exhibit reduced Wnt/β-catenin signaling, delayed mechanical coupling, impaired myofibrillogenesis, and diminished calcium wave velocity, providing key insights into how pathogenic mutations disrupt cell junction assembly and functional integrity.

Although these methods incorporate structural and mechanical features of cardiac tissue, they fall short of recapitulating the complex ECM architecture of the myocardium. Addressing this limitation, Blazeski et al[49] used decellularized porcine ECM slices as a substrate for culturing hiPSC-CMs carrying a *PKP2* mutation (p.A325fs) (Figure 3D). This platform supported the formation of aligned, multilayered syncytia, enabling the study of electrical conductivity and arrhythmogenesis in the context of ACM. Their findings showed significant dysregulation of ACM-related genes in hiPSC-CMs cultured on this ECM substrate compared with traditional monolayers, thereby amplifying the disease phenotype. This advanced model integrates matrix-specific cues to provide a physiologically relevant platform for exploring ACM pathophysiology and functional impairments.[49] In addition, Zhang et al[50] developed larger ECM islands on polyacrylamide gels containing fluorescence beads for traction force microscopy in which multiple hiPSC-CMs carrying *PKP2* truncating variant (p.D109Afs*10) attach and form a 2D multicellular patch (Figure 3E). Through time-lapse imaging, they observed that the pathogenic variant in *PKP2* compromised the dynamics of *CDH2* turnover at the intercalated disc, thus resulting in a significantly diminished pool of *CDH2* available for junction remodeling. This has been shown to markedly destabilize the desmosomes and reduce cardiac contractility.[50]

Altogether, these studies highlight the importance of diverse stimulation modalities in modeling ACM through the utilization of hiPSC-CMs, thereby offering insights into disease mechanisms and potential therapeutic approaches.

HiPSC-EPI

Besides CMs, epicardial cells are considered to play an important role in the development of ACM as a potential source of the fibroadipogenic tissue typical of the disease. To study this hypothesis, Kohela et al[40] used hiPSCs-EPIs carrying the *PKP2* p.Y672Rfs*12 variant. Interestingly, these cells had increased potential for adipogenic and fibrogenic differentiation. In a study by Yuan et al,[51] the authors used single-cell RNA sequencing (RNA-seq) to identify the paracrine role of the epicardium in ACM. They showed that epicardial cells release factors that affect the apoptosis and adipogenesis in CMs, suggesting that paracrine signaling plays a role in explaining the function of epicardium in the modulation of the myocardial environment in ACM.[51] Taken together, these studies have changed the concept of epicardial cells as inert spectators toward active contributors in the pathologic process of ACM. An elaborate understanding of the role of epicardial cells in disease might offer a unique strategy to target the epicardium for treatment of the disease.

HiPSC-derived stromal cells

Cardiac mesenchymal stromal cells play a crucial role in maintaining heart function and promoting repair after injury. These multipotent cells can develop into different cell types (eg, bone, cartilage, and fat cells). CFs are a specific type of stromal cell responsible for producing ECM components such as collagen, elastin, and fibronectin.[52] CFs play a significant role in the pathogenesis of ACM, particularly in regulating fibrofatty remodeling, thereby disrupting the normal electrical conduction pathways in the heart, leading to arrhythmias.[41] Moreover, Wang et al[53] demonstrated that CFs within cardiac scar tissue can directly regulate cardiac excitability and promote arrhythmogenesis, highlighting their active role in the development of arrhythmias. Maione et al[41] pointed out that hiPSC-derived stromal cells can mimic several important phenotypic characteristics of ACM, including fibrofatty tissue accumulation, altered ECM composition, and increased adipogenic differentiation. These findings offer valuable insights into the mechanisms by which stromal cells contribute to the disease phenotype.

Advances in ACM model complexity: 3D cultures

Advancements in traditional 2D cell culture systems have significantly enhanced our understanding of the cellular mechanisms underlying ACM. However, these systems are limited in their ability to replicate the intricate and dynamic mechanical environment of cardiac tissue. Technological progress has enabled the development of advanced in vitro models that more accurately mimic the in vivo cardiac microenvironment. Key innovations in the field of ACM modeling include not only engineered 2D cultures, but also microtissues (MTs), engineered heart tissues (EHTs), and computational modeling frameworks. This section explores the state-of-the-art advances in 3D model systems (Figure 3).

MTs

The concept of culturing cell aggregates in a 3D environment, rather than as 2D monolayers, was first introduced in 1956 by Ehrmann and Gey, using human cell lines embedded in rat tail collagen. Today, the cultivation of cellular aggregates has become an established technique for studying development, homeostasis, and pathology in a 3D context. In the cardiac field, these 3D in vitro models, often referred to as cardiac mini-tissues or MTs, offer valuable insights into the structural and functional dynamics of the heart (Figure 3F).

The first reported ACM MT was generated from hiPSCs derived from a patient carrying a PKP2 mutation (p.Y672Rfs*12).[54] In this study, control MTs and ACM MTs were constructed by combining control hiPSC-CMs and hiPSC-ECs with either control hiPSC-CFs or ACM hiPSC-CFs. The inclusion of ACM hiPSC-CFs significantly impaired the ability of the MTs to respond to high stimulation

frequencies (≥2 Hz), leading to the development of arrhythmic MTs. Moreover, a higher portion of cells positive for α-smooth muscle actin, a well-established myofibroblast marker, was observed in MTs containing ACM hiPSC-CFs compared with controls.[54] These findings demonstrated the value of MTs in modeling complex diseases such as ACM and highlighted the critical role of nonmyocytes, particularly CFs, in the pathogenesis of ACM.

More recently, similar ACM MTs were compared with explanted heart tissues from human patients with ACM and knockin mice carrying mutations in the *PKP2* gene (p.K672Rfs*12), revealing consistent ACM pathologic features across models.[55] In particular, ACM MTs exhibited reduced *PKP2* expression and a marked inability to maintain proper pacing under high-frequency stimulation. These studies collectively underscore the utility of 3D MTs in advancing our understanding of ACM and its underlying mechanism.

EHTs/engineered heart muscles

EHTs, or engineered heart muscles, represent a versatile platform for modeling the biomechanical and electrophysiological properties of cardiac tissues, offering a higher degree of physiological relevance than traditional 2D cultures and 3D MTs (Figure 3G). Since their introduction as force-generating constructs in the 1990s,[56] EHTs have undergone significant advancements in complexity, enabling researchers to investigate the intricate pathophysiology of conditions such as ACM.

Initial EHT models provided valuable insights into the pathologic mechanisms underlying ACM-associated genetic mutations. Ng et al[57] developed one of the first EHT models of ACM by seeding *DSP*-mutated (p.R451G) hiPSC-CMs into decellularized porcine myocardial tissue attached to polytetrafluoroethylene clips. This system enabled the examination of electromechanical coupling in ACM tissues. *DSP*-mutated EHTs exhibited increased connexin-43 phosphorylation, conduction velocity, and time-to-peak compared with wild-type controls, suggesting the absence of arrhythmogenic markers under these conditions.[57]

A similar setup was used by Zhang et al[50] who constructed fibrin-based EHTs suspended between 2 micropillars, allowing quantification of contractile force. Using *PKP2*-mutated hiPSC-CMs (p.D109Afs*10), they observed that, although single cells produced higher systolic stress than wild-type cells, the multicellular EHTs exhibited compromised systolic force generation. This discrepancy was attributed to defective cell-cell adhesion, emphasizing the importance of studying ACM mutations in a tissue-level context.[50]

To incorporate physiological mechanical cues, researchers have developed EHTs that simulate static mechanical conditions such as preload or afterload (Figure 3H). Building on their foundational work, Ng et al[58] composed EHTs of decellularized porcine myocardium seeded with hiPSC-CMs carrying a *DSP* mutation (p.R451G). These EHTs were subjected to 3 static strain regimens using a bioreactor: (1) physiological shortening, (2) increased diastolic stretch (high preload), and (3) isometric culture (high afterload). Remarkably, increased diastolic stretch in *DSP*-mutated tissues led to reduced conduction velocity and connexin-43 expression—effects absent under physiological shortening or isometric conditions. These findings revealed a novel mechanotransduction pathway involving lysosomal activity and ERK signaling, emphasizing the utility of mechanical stimulation to unmask ACM-related phenotypes in EHTs.[58]

Dynamic mechanical conditioning represents the most advanced iteration of EHT complexity, enabling more comprehensive modeling of the dynamic myocardial environment in vivo. Using dynamically stimulated EHTs seeded with *DSP*-mutated hiPSC-CMs (c.273+5G>A and p.R2229Sfs*32), Bliley et al[59] observed hallmark ACM phenotypes, such as contractile dysfunction and arrhythmias, that were absent in unstimulated tissues. Similarly, Simmons et al[60] used micro-heart muscle arrays composed of *PKP2* knockout hiPSC-CMs, which did not require exogenous ECM. These micro-heart muscle arrays revealed that prestretch and cellular alignment were essential for sodium channel function, and they successfully captured ACM-specific defects, including reduced conduction velocity, impaired sodium currents (I_{Nas}), and decreased connexin-43 expression in *PKP2* knockout tissues.[60]

Altogether, these findings reinforce the critical role of mechanical stimulation in modeling ACM and highlight the limitations of static systems in providing a comprehensive view of disease pathology.

Computational models

Although early mathematical models were constrained by limited experimental data, the first computational model of hiPSC-CM electrophysiology was developed in 2013,[61] marking the beginning of a new era in hiPSC-derived cardiac models. Since then, several in silico models have been developed to simulate the electrophysiological and biophysical behaviors of hiPSC-CMs.[62–64] Particularly, Kernik et al[63] developed a model that comprehensively captures the inherent heterogeneity of hiPSC-CM populations, facilitating the study of a broad spectrum of cellular behaviors through the variability of single ion channel current responses.[41] Although computational modeling of hiPSC-CMs remains in its early stages, the focus of the current literature largely revolves around 3 areas: (1) the development and characterization of biophysical models of hiPSC-CMs,[62,63] (2) the calibration and customization of these models for individual hiPSC-CM lines,[64] and (3) the use of such models for genetic mutation risk prediction.[63]

To the best of our knowledge, Parrotta et al[65] are the first to publish an in silico model of ACM in hiPSC-CMs, aimed at investigating the molecular dynamics that drive heart remodeling and the loss of CM identity during ACM (Figure 3I). Their work presents a mathematical model describing the interplay between the Wnt pathway and the Ras homolog family member A (RhoA)–Rho-associated protein kinase (ROCK) pathway, both of which are crucial in the pathogenesis of ACM.[66,67] By

comparing their model with experimental data from hiPSC-CMs derived from both healthy individuals and patients with ACM, they demonstrated not only that desmosome stability is crucial for regulating adipogenesis but also that activation of both Wnt and RhoA-ROCK pathways inhibits adipogenesis, a key event in ACM progression.[65]

The continued advancements of hiPSC-CM-based computational models hold significant promise for enhancing our understanding of ACM pathophysiology and developing targeted therapeutic strategies. As computational models become more refined and data availability improves, they will likely play an integral role in both preclinical research and clinical trials, enabling the development of more effective and individualized treatments to prevent or mitigate ACM onset and progression.

Mechanistic insights from hiPSC-based ACM models

Phenotypic manifestations of ACM include electrical abnormalities, fibrotic remodeling, impaired contractility, and inflammation, all of which are integral to disease progression. Traditional models, including animal studies and patient-derived tissue analyses, have provided valuable insights but are limited in their ability to replicate the patient-specific molecular and cellular mechanisms underlying ACM. In contrast, hiPSC-based models can recapitulate key features of ACM in a patient-specific and therefore mutation-specific manner. These models have been instrumental in dissecting the genetic, molecular, and electrophysiological mechanisms of ACM and evaluating novel therapeutic interventions. In this section, we explore how hiPSC-based 2D and 3D models have been used to reproduce distinct ACM features, highlighting the mechanistic insights gained and the experimental methodologies used.

Modeling adipogenesis

Adipogenesis, characterized by the replacement of healthy myocardium with fibrofatty tissue, is a hallmark of ACM.[68] Despite its significance, the precise origins of adipocytes and the molecular mechanisms driving adipogenesis in ACM remain poorly understood. Notably, the replication of adipogenesis in murine models is highly challenging,[32] underscoring the necessity for developing and using in vitro systems to facilitate the investigation of this phenotype.

Mechanistic insights from hiPSC-based ACM models

The earliest studies using hiPSC-based models to investigate adipogenesis in ACM identified pathologic fatty infiltration as a key phenotype. Kim et al[69] and Ma et al[70] explored this phenomenon in hiPSC-CMs with *PKP2* mutations (c.2484C>T and p.L614P, respectively), using adipogenic induction medium to create an adipogenic environment. Both groups reported exaggerated lipid accumulation and apoptosis in mutated hiPSC-CMs compared with controls. Kim et al[69] additionally described an upregulation of adipogenic genes, including *PPARγ* and *FABP4*, in *PKP2*-mutant cells. Intriguingly, they demonstrated that activation of *PPARγ* alone (via application of rosiglitazone or indomethacin) did not induce excessive lipid production, suggesting that dysregulation of both PPARγ and PPARα pathways was necessary to induce an adipogenic phenotype.

Further studies reinforced the role of lipid accumulation in ACM pathogenesis. Blazeski et al[49] demonstrated that hiPSC-CMs carrying a *PKP2* mutation (p.A325fs) developed lipid and neutral fat droplet accumulation when cultured on engineered heart slices composed of ACM porcine ECM, as confirmed by perilipin A and Nile red staining. These findings highlight the influence of the extracellular environment in promoting adipogenesis in ACM.

Although desmosomal protein mutations are central to ACM pathology, recent evidence suggests that nondesmosomal gene mutations linked to ACM may also drive adipogenesis. Chen et al[24] investigated hiPSC-CMs with *OBSCN* mutations (p.L5218Sfs*25), a structural protein linking sarcomeres to the sarcoplasmic reticulum,[71] and observed significant lipid accumulation, increased fibrofatty area, and upregulation of adipogenesis-related pathways. Using transcriptomic analyses validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting, they identified elevated expression of PPARγ, C/EBPα, and FABP4 proteins, implicating these pathways in the adipogenic phenotype of OBSCN-mutant hiPSC-CMs.[24]

Lipogenesis was also observed in a study aiming to determine the effects of sex hormones in ACM hiPSC-CMs carrying the c.2484C>T mutation.[72] The findings of this study indicated that testosterone exacerbated CM apoptosis and lipogenesis, whereas estradiol exerted a protective effect. These findings suggest that hormonal factors may play a significant role in ACM progression.[73]

Origins of adipocytes in ACM

In addition to molecular drivers, the cellular origin of adipocytes in ACM has been a topic of investigation. Kohela et al[40] examined hiPSC-EPIs from patients with ACM (*PKP2,* p.Y672Rfs*12), using single-cell RNA-seq to reveal spontaneous fibrofatty differentiation absent in isogenic controls. They identified the transcription factor AP-2 alpha as a key regulator, promoting epithelial-to-mesenchymal transition and adipogenic differentiation in ACM epicardial cells,[40] indicating the capability of epicardial cells to contribute to the population of adipocytes in ACM.

Moreover, Maione et al[73] explored the adipogenic potential of cardiac mesenchymal stromal cells derived from hiPSCs (hiPSC-D) using Nile red staining and qPCR. They demonstrated that ACM hiPSC-D cells exhibited a higher propensity for lipid droplet accumulation and upregulation of adipogenic genes than healthy controls, underscoring the contribution of stromal cells to fibrofatty remodeling in ACM.[73]

Computational approaches to investigate adipogenesis in ACM

Complementing experimental studies, computational modeling has provided valuable insights into the regulatory

dynamics of adipogenesis in ACM. Parrotta et al[65] developed a mathematical model to examine the interplay between Wnt/β-catenin and plakoglobin, demonstrating their competitive influence on adipogenic marker expression. They further investigated the RhoA-ROCK pathway and its crosstalk with Wnt signaling, concluding that simultaneous inactivation of Wnt/β-catenin and RhoA-ROCK pathways is required for significant PPARγ upregulation.[65] These findings underscore the complexity of adipogenesis regulation in ACM.

Modeling arrhythmogenesis

Arrhythmogenesis, the development of abnormal electrical activity leading to arrhythmias, is a major clinical manifestation of ACM.[5] It often precedes structural changes in the myocardium and contributes significantly to the risk of SCD.[5,6] However, the complex interplay among structural remodeling, desmosomal dysfunction, and electrophysiological abnormalities in ACM remains incompletely understood.

Electrical abnormalities in ACM

Electrical abnormalities in hiPSC-CMs were first reported by Kim et al,[69] who demonstrated impaired calcium (Ca^{2+}) handling in hiPSC-CMs carrying a *PKP2* mutation (c.2484C>T). Using Ca^{2+} imaging and qRT-PCR, they identified decreased levels of sarcoplasmic reticulum Ca^{2+}-ATPase, a key regulator of intracellular calcium dynamics.[74] These findings established a foundational link between genetic mutations and disrupted calcium homeostasis in hiPSC-CMs.[69] Similar abnormalities in calcium regulation were later observed in hiPSC-CMs with FLNC knockout.[46]

Further investigation into electrical dysfunctions was performed by El-Battrawy et al,[43] who studied hiPSC-CMs carrying a missense *DSG2* mutation (p.G638R). The authors used calcium imaging, qRT-PCR, and patch clamp techniques, revealing multifaceted ion channel dysfunctions, including alterations in sodium channel activity and Na^{+}/Ca^{2+} exchanger performance. These cellular electrophysiological abnormalities were exacerbated by adrenergic stimulation.[43] Moreover, Gusev et al[44] studied DSP p.H1684R mutant hiPSC-CMs and documented reduced I_{Na} and L-type calcium currents along with shortened action potential duration at 50% amplitude, indicating the combined effects of ion channel dysregulation on action potential modulation. In the same year, Chen et al[24] studied *OBSCN* mutations in hiPSC-CMs and observed irregular L-type calcium current density and disordered ion channel function, contributing to electrical instability. In addition, Khudiakov et al[75] studied *PKP2*-mutant hiPSC-CMs (p.Y119Mfs*23 and p.K859R) and found significant reductions in I_{Na} density and action potential upstroke velocity. Notably, these changes were rescued by wild-type *PKP2* transduction, highlighting the critical role of PKP2 in maintaining normal electrical activity. Furthermore, Moreau et al[45] studied *DSC2* p.R132C mutant hiPSC-CMs and found reduced I_{Na} density, increased repolarizing currents, and shortened contraction duration, accompanied by impaired calcium handling. In addition, optical mapping of *DSG2*-mutated hiPSC-CMs revealed decreased action potential amplitudes, increased upstroke heterogeneity, and reduced time-to-peak calcium and slower calcium decay rates. These phenomena were accompanied by dysregulated expression of ion channels and calcium-handling genes.[76]

Recent studies have provided mechanistic insights into the molecular basis of these electrical abnormalities, particularly those caused by *PKP2* mutations. Kim et al[47] demonstrated that the heterozygous PKP2 variant p.R413X reduced Wnt/β-catenin signaling, delayed mechanical coupling, impaired myofibrillogenesis, and decreased calcium wave propagation. Their use of EHTs offered a more physiologically relevant 3D model for investigating these abnormalities. Restoration of Wnt/β-catenin signaling was found to improve cell junction integrity and calcium wave velocity, highlighting both the pathway's critical role in maintaining electrophysiological homeostasis and its potential as a therapeutic target.

Complementary findings by Simmons et al[60] revealed that *PKP2* knockout hiPSC-CMs exhibit significantly reduced conduction velocities and a complete absence of functional I_{Nas}. These findings suggest that impaired ion channel–gap junction interactions underlie the decreased conduction velocity observed in ACM-affected tissues.[60]

Adding another layer of complexity, Ng et al[58] investigated the effects of mechanical stress on 3D EHTs composed of DSP-mutated hiPSC-CMs (p.R451G). Under cyclic strain, these tissues showed reduced conduction velocities and decreased connexin-43 expression, particularly with increased diastolic stretch. Remarkably, these impairments were reversible through the inhibition of lysosomal activity and ERK signaling, implicating ERK signaling as a key regulator of conduction velocity under mechanical stress conditions.[58]

Contractile dysfunction in ACM

Electromechanical coupling, the process by which electrical signaling in CMs drives mechanical contraction, is essential for effective cell and tissue function. Ng et al[57] investigated the effects of *DSP* mutations on conduction velocity and contractile performance in linear tissue constructs composed of hiPSC-CMs. They observed a significant prolongation of the time-to-peak contraction in *DSP*-mutated tissues compared with wild-type controls. Notably, this delay in contraction kinetics occurred without significant differences in peak force or time to 50% relaxation, suggesting that *DSP* mutations impair contraction timing rather than the overall force generated by the tissue.

Building on these findings, Bliley et al[59] explored DSP-mutated hiPSC-CMs in EHTs subjected to cyclic loading, simulating mechanical stress. In contrast to Ng et al,[57] they observed faster contraction kinetics accompanied by a significant reduction in contractile force amplitude and twitch stress. The discrepancies between these studies may reflect differences in the specific mutations studied or experimental

conditions, particularly the application of increased diastolic stress in the cyclic loading model, which likely amplified the contractile impairments in mutant tissues.[59]

These findings highlight the nuanced effects of *DSP* mutations on contraction kinetics and force generation in hiPSC-CM models. Although *DSP* mutations consistently disrupt contraction timing, the magnitude of their impact on contractile force seems to depend on the mechanical environment, underscoring the importance of considering external stress conditions in studying ACM pathophysiology.

Modeling fibrosis

Fibrosis, characterized by the replacement of healthy myocardial tissue with fibrotic tissue, is a hallmark of ACM. Several studies using hiPSC-based ACM models have demonstrated similar fibrotic remodeling behaviors and identified shared molecular features underlying this pathologic process.

Role of hiPSC-CMs in fibrotic remodeling

Martewicz et al[47] investigated the effects of dysfunctional desmosomes on fibrofatty remodeling in hiPSC-CMs carrying a *PKP2* mutation under mechanical stimulation (cell patterning combined with cyclic stretch). RNA-seq revealed significant transcriptional changes indicative of a profibrotic gene expression program. In particular, they observed the downregulation of fibril-associated collagens with interrupted triple helices, which are associated with maintaining ECM structural integrity,[77] and a strong upregulation of fibril-forming collagens, fibronectin, and other profibrotic markers such as TIMP1. These findings highlight a profound shift in ECM composition and ECM-interacting proteins in *PKP2*-mutated hiPSC-CMs, providing insights into the fibrotic remodeling response.[47]

Role of non-CMs in fibrotic remodeling

In addition to hiPSC-CMs, other ACM-relevant cell types contribute to fibrosis. Kohela et al[40] studied hiPSC-EPIs carrying a *PKP2* mutation and observed a downregulation of collagen degradation genes and an upregulation of fibroblast activation markers. Moreover, these cells exhibited a loss of the epicardial cell marker WT1 and a significant induction of fibrosis markers after prolonged culture (80 days), suggesting a transition toward a fibrofatty cell identity. This transition was shown to be mediated by the transcription factor AP-2 alpha, which not only promotes adipogenesis but also contributes to fibrotic pathways,[78] underscoring its role in epicardial remodeling in ACM.[47]

Further contributions to fibrosis were identified in hiPSC-D carrying p.K672Rfs*12 and p.G548Vfs*15 *PKP2* mutations. Maione et al[73] reported that these cells exhibited an increased propensity for collagen accumulation, as evidenced by immunofluorescence staining for collagen I, further implicating stromal cells in the fibrotic process of ACM.

These studies collectively demonstrate that fibrosis in ACM is a multicellular process involving contributions from CMs, epicardial cells, and stromal cells. However, the specific role of ACM-affected CFs, the key player in fibrosis, in this process remains unexplored, representing a critical gap in understanding the fibrotic remodeling mechanisms in ACM.

Modeling inflammation

Myocardial inflammation is a frequent finding in biopsies from patients with ACM, suggesting its involvement in ACM pathology. However, whether inflammation is a primary cause or a secondary consequence of the disease remains unclear.[79] Investigations into inflammation using hiPSC-CM models of ACM are limited, with only 2 studies to date addressing this aspect.

Chelko et al[80] provided the first insights into the inflammatory mechanisms in ACM by characterizing nuclear factor-kappa B (NF-κB) signaling in hiPSC-CMs carrying a *PKP2* mutation (p.K672Rfs*12). They assessed cytokine production and the nuclear accumulation of phosphorylated RelA/p65 (Ser536), a key marker of NF-κB pathway activation, both under basal conditions and after exposure to inflammatory stimuli. Their findings revealed constitutive NF-κB activation in *PKP2*-mutant hiPSC-CMs, accompanied by elevated cytokine expression and secretion under basal conditions. Treatment with the NF-κB inhibitor BAY 11-7082 markedly reduced cytokine levels in both the cells and the culture medium, while also preventing phosphorylated RelA/p65 nuclear translocation. These results highlighted that *PKP2*-mutant hiPSC-CMs exhibit an active innate immune response driven by NF-κB signaling.[80]

Building on this work, Hawthorne et al[76] extended the investigation to hiPSC-CMs with *DSG2* mutations. They observed significantly increased expression of proinflammatory cytokines and chemokines in *DSG2*-mutant cells compared with wild-type controls. Notably, these upregulated factors included members of the interleukin family and adipofibrokines, which play roles in immune cell recruitment, fibrotic remodeling, adipocyte survival, proliferation, and lipid accumulation.[76]

To gain deeper insights into the molecular pathways involved, the same authors performed bulk RNA-seq combined with the Kyoto Encyclopedia of Genes and Genomes pathway analysis. They identified upregulation of 4 cell surface receptors involved in NF-κB signaling in *DSG2*-mutated hiPSC-CMs: *CD40* and *XEDAR* (noncanonical pathway) and *IL1R* and *TLR4* (canonical pathway). This dual activation suggests that both canonical and noncanonical NF-κB pathways contribute to the inflammatory response observed in ACM hiPSC-CMs.[76]

Together, these studies underscore the role of NF-κB signaling in mediating inflammation in ACM, demonstrating that hiPSC-CMs carrying ACM-related mutations exhibit heightened innate immune responses. These findings also

highlight potential therapeutic targets, such as NF-κB pathway inhibitors, for modulating inflammation in ACM.

Pathway alterations

ACM pathogenesis is complex and yet not fully defined. Different studies in animal models suggest the involvement of the Wnt/β-catenin, Hippo, and transforming growth factor β pathways.[67,81–83] However, whether these alterations occur also in humans and which additional pathways contribute to ACM remain unclear. Alterations in the Wnt/β-catenin signaling have also been identified as key contributors to ACM pathogenesis in hiPSC-CMs by Khudiakov et al.[75] The authors demonstrated that *PKP2* p.Y119Mfs*23 and p.K859R mutations did not affect Wnt/β-catenin signaling activity in undifferentiated ACM-hiPSCs. However, during differentiation, β-catenin activity was notably reduced in mutant-derived hiPSC-CMs compared with controls, suggesting that these alterations emerge during CM maturation in the presence of pathogenic variants.[75] More recently, in 2023, de Bortoli et al[84] provided further evidence of Wnt signaling dysregulation in *PKP2*-mutated hiPSCs-CMs. In this study, *PKP2* p.N346Lfs*12 ACM-hiPSC-CMs and asymptomatic hiPSC-CMs exhibited significantly lower levels of active β-catenin than control cells. This observation highlighted the disruption of Wnt signaling as a hallmark feature in ACM pathogenesis.

Additional research to unravel the molecular mechanisms underlying ACM was performed in 2 different iPSC-CM lines carrying mutations in the *FLNC* gene. In 1 study, RNA-seq performed on FLNC p.E2189* iPSC-CMs revealed upregulation of genes associated with platelet-derived growth factor binding and activation of ERK signaling.[85] A different study, focused on the p.R1267Q variant in the same gene, identified the suppression of pathways related to myofibril assembly, cardiac contraction, and ECM organization.[86]

Limitations of iPSC-based models

Although hiPSC-based models have significantly advanced our ability to study ACM, they are not without limitations.

First, the vast majority of ACM studies and discoveries to date were done on models carrying *PKP2* variants. To overcome this limitation, it is essential to generate a wider variety of models encompassing mutations in other ACM affected genes (eg, *DSP*, *DSG2*, *JUP*) and representing recessive forms of ACM, such as Naxos disease and Carvajal syndrome, caused by homozygous mutations in *JUP* or *DSP* genes, respectively.[16,87] This would result in a more comprehensive understanding of the phenotype of ACM, enhance the genotype-phenotype correlation, and support the development of precision medicine tools.

Furthermore, hiPSC-based models face challenges related to cellular immaturity, heterogeneity, and the limited ability to recapitulate complex tissue-level features. hiPSC-CMs, the key hiPSC-based ACM model, display immature structural and electrophysiological properties compared with adult CMs. These include a poorly developed sarcoplasmic reticulum, absence of transverse tubules, and markedly reduced expression of the inward rectifier potassium current, even after prolonged culture.[88] This evidence explains their depolarized maximum diastolic potential or resting membrane potential. In addition, hiPSCs-CMs have been shown to express both fetal and adult isoforms of SCN5A, whereas adult cardiac tissue expresses only the adult isoform.[89] These limitations could be mitigated by increasing the use of 3D models and incorporating different cardiac cell types. This approach, on the one hand, could improve hiPSC-CM maturation and, on the other hand, provide a more suitable platform to investigate intercellular crosstalk and additional disease features. In particular, the inclusion of fibroblasts and immune cells may enable the modeling of fibrosis, inflammation, and/or adipogenesis, which are key features of ACM poorly modeled in current in vitro systems.

Finally, although hiPSC-based models offer important advantages, such as accessibility to molecular and functional readouts and fine control over experimental parameters, they still fail to fully recapitulate the in vivo physiological environment of the human heart in vivo. A stronger focus on the comparison between hiPSC-based and in vivo models might reveal shared features or model-specific aspects. Similar alterations in desmosomal protein expression and contractile function were detected in in vitro hiPSC-CMs or engineered heart muscle and an in vivo mouse models harboring the same pathogenic variant in *PKP2*.[90] Advancing this field will be key to ensuring a smoother and more predictive transition from in vitro findings to in vivo validation in drug testing.

Future perspectives

Improvements of in vitro models to study ACM

This review explores recent advances in in vitro models for ACM and their ability to replicate key pathologic features and uncover disease mechanisms. Compared with in vivo models, hiPSC-based approaches provide a scalable, patient-specific platform for investigating disease mechanisms and conducting drug screening for potential therapies, reducing reliance on in vivo testing. In this section, we highlight the current challenges and future directions for hiPSC-based models of ACM (Figure 4).

Recent technological developments have significantly advanced in vitro ACM modeling. Although 2D monolayers and 3D MTs have provided essential insights, more sophisticated systems, such as organoids and heart-on-a-chip platforms, remain largely unexplored in ACM research, presenting promising future directions.

Cardioids, a specific type of cardiac organoid, replicate the cellular composition and function of the human heart. A defining characteristic of cardioids is their self-organization capability, allowing them to develop tissue-like structures and functional properties similar to adult cardiac tissue. Currently, they are primarily used to study cardiac development.[91–93] However, recent studies suggest their broader applicability in disease modeling: Lewis-Israeli et al[94] demonstrated their potential in metabolic disorders, whereas

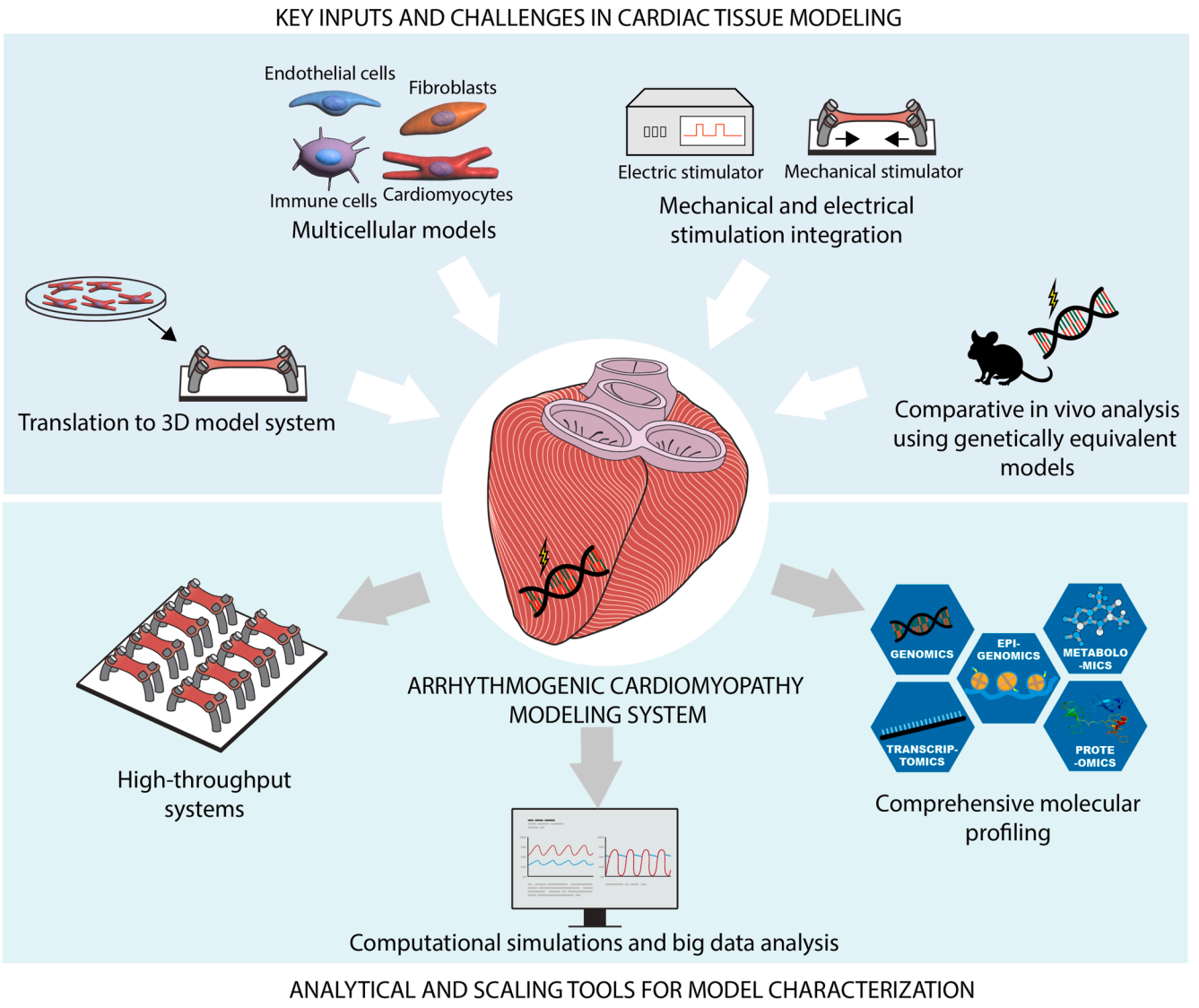

Figure 4
Current challenges and future directions for hiPSC-based models of arrhythmogenic cardiomyopathy (ACM). Future improvements should include (1) the development of three-dimensional (3D) culture systems; (2) integration of multicellular interactions (eg, cardiomyocytes, fibroblasts, immune cells, and endothelial cells); (3) application of mechanical and electrical stimulation to simulate the dynamic cardiac niche; and (4) comparative in vivo validation using genetically matched animal models. In addition, these platforms should support high-throughput experimentation and enable deep molecular profiling. Combined with computational models, such integrative approaches will help elucidate disease mechanisms, uncover novel therapeutic targets, and guide regenerative strategies for ACM. 3D = 3-dimensional; hiPSC = human-induced pluripotent stem cell.

Volmert et al[95] used cardioids to test drug effects on congenital heart defects. Given their ability to closely mimic in vivo cardiac tissue, cardioids could provide a powerful new model for studying ACM. Their self-organization properties may allow researchers to investigate disease hallmarks such as fibrofatty replacement and electrophysiological abnormalities, which remain difficult to model in conventional systems.

One key limitation of current 3D in vitro ACM models is the lack of vascularization, which is crucial for accurately modeling nutrient exchange, drug delivery, and cell-cell interactions. Most ACM research has focused on hiPSC-CMs, with more recent studies incorporating hiPSC-EPIs and hiPSC-CFs. Although hiPSC-ECs have been implicated in genetic cardiomyopathies, particularly dilated cardiomyopathy, their role in ACM remains underexplored, representing a significant gap in model development.[96] In addition, the absence of vascularization, immune cells, and neural-derived cells in current 3D models limits their ability to fill the gap and fully recapitulate ACM pathology, emphasizing the need for next-generation models that integrate these components. Vascularized MTs address this issue by incorporating endothelial networks, mimicking the microvascular environment. For example, Arslan et al[97] developed a vascularized cardiac MT model that enables the study of drug-endothelium interactions. Using a similar model, Landau et al[98] studied the role of macrophages and observed improved vascularization in MTs, significantly improving physiological relevance.

Heart-on-a-chip technologies integrate cellular tissues with microfluidic elements, environmental controls (eg, mechanical and electrical stimulation), and real-time analytical components, offering an advanced platform for modeling pathogenic conditions. Such systems have been successfully used to study myocardial infarction, as demonstrated by Veldhuizen et al,[99] who investigated contraction and metabolic alterations owing to oxygen level fluctuations. Similarly, heart-on-a-chip platforms have been used to model dilated cardiomyopathy, revealing key disease features such as altered I_{Na} currents and reduced contraction force under chronic electrical stimulation.[100]

Applying similar approaches to ACM models could help assess the impact of vascular dysfunction on disease progression and therapeutic responses but also provide insights into how mechanical and electrical stress contribute to disease progression.

Enhancing mechanical and electrical stimulation

Automated electrical stimulation and biosensing platforms can significantly improve hiPSC-CM maturation, reproducibility, and scalability, ultimately reducing reliance on animal models. A promising example is the integration of optical fibers into multiwell plates, as demonstrated by Gracioso Martins et al,[101] which enables real-time monitoring of beating activity across all wells of a 96-well plate. Furthermore, the development of automated pipelines for action potential analysis, such as the system proposed by Soepriatna et al,[33] represents a step forward in standardizing data collection.

However, achieving reliable readouts still requires further advancements. The addition of both electrical and mechanical stimulation can help standardize experimental conditions, avoiding the variability associated with spontaneous beating. For instance, Jayne et al[102] developed a system combining mechanical stimulation with electrical sensing to assess contractile forces in MTs. Similarly, Zhao et al[103] introduced a platform capable of measuring Ca^{2+} transients, active force, conduction velocity, and action potentials continuously. Their system also allows chronic electrical stimulation of MTs for up to 8 months, providing a model for stress-like conditions and phenotype exacerbation (particularly in left ventricular hypertrophy).[103] Although some high-throughput scalable platforms integrating electrical stimulation and biosensing have been developed in 2D cultures, significant opportunities remain for further advancements in 3D models.[104]

To address these challenges, future research should focus on the development of bioengineered scaffolds, organ-on-a-chip platforms, and vascularized cardiac MTs to improve physiological relevance and on optimizing these models to balance complexity with practicality to ensure wider application in ACM studies and drug development. Given that in vitro models become increasingly complex, the establishment of high-throughput systems and multi-omics approaches will be essential to advance the field.

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