Minireview

Bioengineering approaches to mature induced pluripotent stem cell-derived atrial cardiomyocytes to model atrial fibrillation

Olivia T Ly[1,2], Grace E Brown[2], Yong Duk Han[2], Dawood Darbar[1,2,3] and Salman R Khetani[2]

[1]Division of Cardiology, Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA; [2]Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60607, USA; [3]Department of Medicine, Jesse Brown VA Medical Center, Chicago, IL 60612, USA

Corresponding author: Salman R Khetani. Email: skhetani@uic.edu

Impact statement

There is currently no comprehensive model of atrial fibrillation (AF) to study human pharmacological response and assess the toxicity of novel drug candidates. The use of patient-specific induced pluripotent stem cell-derived atrial cardiomyocytes (iPSC-aCMs) has been shown to model the electrophysiological disease phenotype and genotype of AF. However, this cellular foundation is immature and does not capture the mature, adult phenotype of aCMs in native myocardium. Although current methods of engineering maturation have evolved, sufficient maturation of iPSC-aCMs has not yet been achieved; in addition, these approaches have primarily been applied to ventricular or heterogeneous cell populations. This review summarizes the current technologies that are being developed that can be applied to the maturation of patient-specific iPSC-aCMs.

Abstract

Induced pluripotent stem cells (iPSCs) serve as a robust platform to model several human arrhythmia syndromes including atrial fibrillation (AF). However, the structural, molecular, functional, and electrophysiological parameters of patient-specific iPSC-derived atrial cardiomyocytes (iPSC-aCMs) do not fully recapitulate the mature phenotype of their human adult counterparts. The use of physiologically inspired microenvironmental cues, such as postnatal factors, metabolic conditioning, extracellular matrix (ECM) modulation, electrical and mechanical stimulation, co-culture with non-parenchymal cells, and 3D culture techniques can help mimic natural atrial development and induce a more mature adult phenotype in iPSC-aCMs. Such advances will not only elucidate the underlying pathophysiological mechanisms of AF, but also identify and assess novel mechanism-based therapies towards supporting a more 'personalized' (i.e. patient-specific) approach to pharmacologic therapy of AF.

Keywords: Atrial fibrillation, human-induced pluripotent stem cell-derived atrial cardiomyocytes, disease modeling, maturation, pharmacologic response

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Introduction

Atrial fibrillation (AF), the most commonly encountered sustained cardiac arrhythmia in clinical practice,[1,2] is associated with significant morbidity and increased mortality. Epidemiological studies have projected that by the year 2050, 12.1 million Americans will be diagnosed with AF; however, this number is likely to increase to ~16 million with the aging of the population and the identification of novel risk factors such as obesity and obstructive sleep apnea.[3] It is estimated that one in three individuals of European descent over the age of 40 will be diagnosed with AF, and this is associated with a considerable increase in risk of stroke, heart failure, dementia, and death.[4,5] The typical symptoms associated with AF include palpitations, presyncope, exercise intolerance, extreme fatigue, chest pain, and shortness of breath with exertion.

Over the last decade, tremendous progress has been made in catheter-based therapies, but antiarrhythmic drugs are still the most commonly used form of therapy for patients with symptomatic AF.[6,7] However, individual response to membrane-active drugs can be associated with serious toxicities and is highly variable, with ~50% of patients experiencing a recurrence of AF within six months.[8] Due to the heterogeneity of the underlying

electrical and structural substrate of AF, as well as the failure to target therapy to the underlying mechanisms, there currently exists a wide range of therapies to treat AF. Genetic approaches to the mechanisms of AF have not only provided important insights into the underlying pathophysiology but also identified novel therapeutic targets.[5,9] However, the direct impact of these genetic discoveries to the bedside care of patients has been limited because the *in vitro* (heterologous expression systems) and *in vivo* (murine, sheep, canine) models established to assess the role of AF-causing ion channels variants do not fully capture the complex array of ion channels in atrial cardiomyocytes (aCMs), or model human AF and pharmacological response.[10,11] The development of a comprehensive modeling system for AF that not only identifies the underlying pathophysiological mechanisms of AF but also enables the screening of mechanism-based therapies would be a major advancement in providing personalized, i.e. patient-specific, care for patients with AF.

Patient-specific models are needed to elucidate the underlying cellular and molecular mechanisms and abnormal electrophysiological (EP) properties of mutations that cause AF. However, access to human atrial tissue is rarely available to harvest primary aCMs, and even if harvested, expanding and maintaining aCMs in sufficient amounts for longitudinal studies and drug screening are nearly impossible. Therefore, patient-derived induced pluripotent stem cell-derived atrial specific cardiomyocytes (iPSC-aCMs) can provide insights into the underlying genetic mechanisms of AF and be useful to explore phenotype–genotype relationships (Figure 1). These cells also express the complex array of cardiac ion channels generating the atrial action potential (AP) and can be electrically coupled to elucidate AF mechanisms.[12,13] Overall, iPSC-aCMs offer distinct advantages over heterologous expressions systems and animal models.

IPSC-aCMs are particularly suited to modeling AF-causing mutations as they elicit cell-autonomous EP phenotypes, and disease-specific iPSC models maintain functional trademarks of the mutation *in vitro*.[14] In addition, correcting the genetic variant with clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9[15]

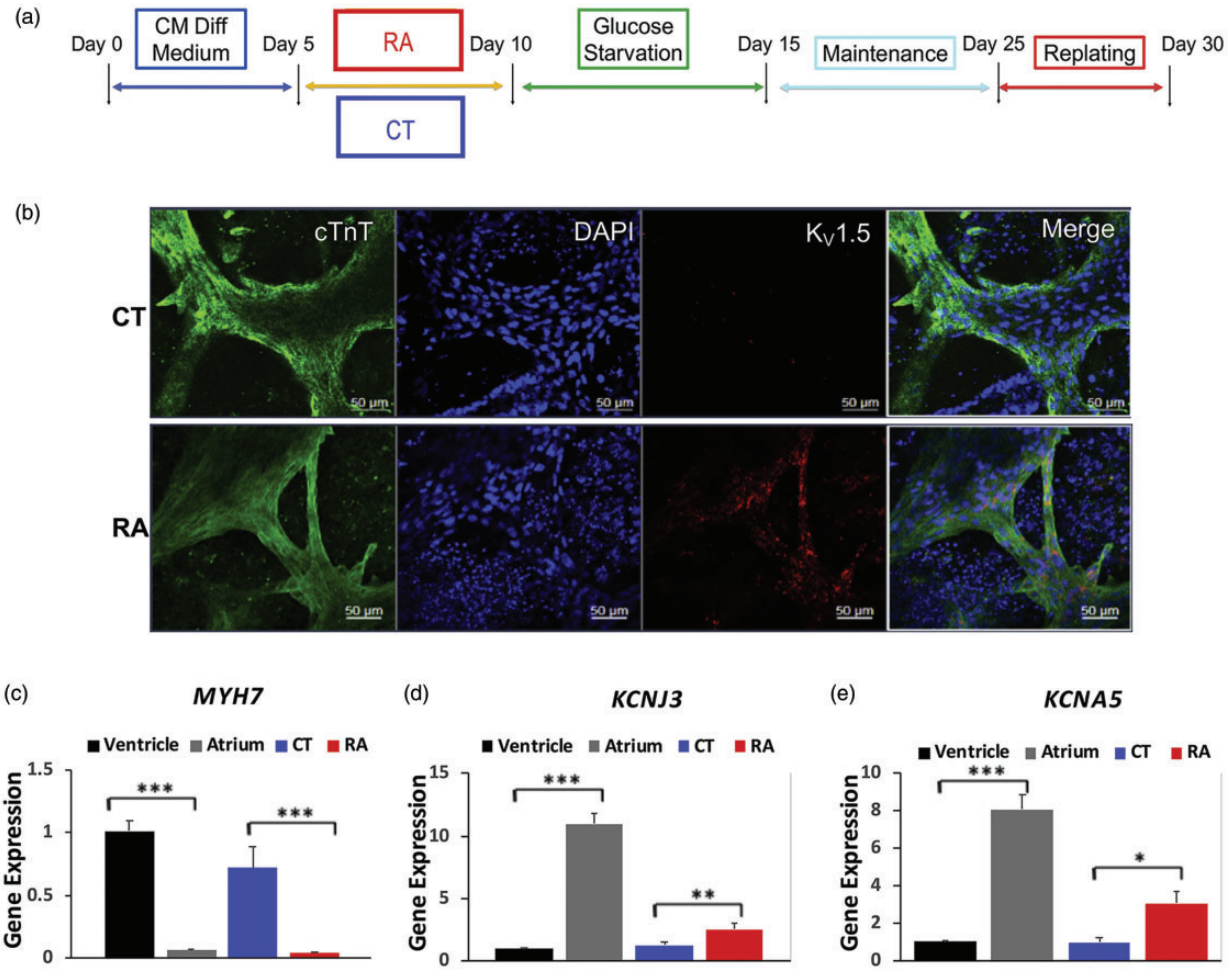


Figure 1. Differentiation of iPSCs to atrial cardiomyocytes: (a) Protocol for differentiation of iPSCs into atrial cardiomyocytes. iPSCs are differentiated into cardiomyocytes using a commercially available cardiomyocyte differentiation medium for 5 days, then incubated with 1 μM retinoic acid (RA) or DMSO (vehicle control or CT) for another 5 days to enrich iPSC-aCMs, and the iPSC-aCMs are further enriched via glucose starvation for 5 more days, for a total of 15 days. (b) Immunostaining showing the protein expression of pan-CM marker cardiac cTnT and atrial marker Kv1.5 in hiPSC-CMs at day 10 comparing RA treated cells to CT cells. (c) qRT-PCR of ventricular marker, MYH7, in RA-treated and CT cells and atrial markers, KCNJ3 and KCNA5, in RA-treated and CT cells at day 30. Figure adapted from Argenziano *et al*.[10] (A color version of this figure is available in the online journal.)

permits assessment of the AF phenotype in different genetic backgrounds, and helps determine human pharmacological responses targeted to the mutation, paving the way for a more "personalized" approach to AF therapy.[16,17] Although iPSC-derived cardiomyocytes (CMs) faithfully model several human arrhythmia syndromes including AF, when compared with adult CMs, their structural, molecular, metabolic, and EP immaturity is a limitation.[18,19] Thus, enhancing the maturity of iPSC-aCMs will not only elucidate the underlying cellular mechanisms of AF, but also help identify signaling pathways that are critical for atrial development.[10,20]

Metrics of CM maturity

Structural and functional assessment

During cardiac development, CMs undergo a series of structural, metabolic, EP, and functional changes that lead to its adult phenotype (Table 1). The structural maturity of CMs is crucial to internal cytoskeletal organization/integrity and functional properties including membrane capacitance, bioenergetics efficiency, excitation–contraction coupling, and conduction velocity. Cell morphology is also critical for maximizing and optimizing cell–cell communication since CMs communicate with each other on the longitudinal edge via gap junctions. Cell size is critical because it influences impulse propagation, conduction velocity, contractile force, and membrane capacitance.[21] Sarcomeres are the contractile units of CMs and are important for the structural integrity, cytoskeletal organization, and contractile strength of CMs. To assist the sarcomeres in propagating the AP from cell to cell, T-tubules, invaginations in the CM sarcolemma between sarcomeres at the Z-disks, transmit AP impulses to the sarcoplasmic reticulum (SR). T-tubules are also responsible for rapid excitation–contraction coupling and synchronous calcium release from the SR to allow the myocardium to function as a syncytium.

Structural and sarcomeric genes are upregulated, and there is an isoform shift to the adult isoform throughout development. Immature iPSC-aCMs, however, lack sufficient expression of general CM markers, such as cardiac troponin T (cTnT; *TNNT*) and cardiac troponin I (cTnI; *TNNI3*). Even when matured, iPSC-aCM and aCMs for that matter share many characteristics with immature ventricular CMs (Table 2). For example, myosin heavy chain (MHC) is responsible for hydrolyzing ATP in energy production and contractile force, and is thus also crucial for maintaining sufficient maximal velocity (V_{max}).[22] Ventricular CMs transition from the α-isoform (MYH6) to the β-isoform (MYH7) during development, whereas the dominant MHC isoform in primary atrial CMs is the α-isoform, resulting in lower overall contractile force production. Myosin light chain (MLC) controls the maximum tension produced by the cells.[23] Ventricular CMs transition from MLC2-a (MYL7) to MLC2-v (MYL2), while MLC2-a dominates in atrial CMs; this is likely the primary reason why atrial CMs produce reduced active tension, reduced resting tension, reduced resting stiffness, and faster rate of sarcomeric shortening than ventricular CMs.[22,23] Titin is expressed in two isoforms: N2B and N2BA. N2B is the larger and more compliant isoform, and is more dominantly expressed in ventricles as ventricular maturation progresses. N2BA, the shorter and stiffer isoform, is

Table 1. Metrics of maturity.

Assessment of maturity	iPSC-aCM	Adult aCM
Structural		
Morphology	Circular	Rod-shaped, length:width = 7:1[18]
Type of growth	Hyperplastic, proliferative	Hypertrophic
Membrane surface area	1000–1300 μm^2 [23]	10,000–14,000 μm^2 [23]
Sarcomere length	1.7 μm	2.2 μm
Sarcomere organization	Unorganized, perinuclear clustering[21–23]	Organization maintained throughout the cell[21–23]
Specialized intracellular organelles	Lacking, or not fully formed	Sarcoplasmic reticulum, T-tubules, sarcomeric subunits (H-, A-, I-bands, Z-disks)
Multinucleation	Primarily mononucleated	25–30% binucleated, up to 8 nuclei
Myofibrillar isoform	*ssTNI MHC-α > MHC-β[29]MLC2-a > MLC2-v*[30]*TTN-N2BA > TTN-N2B*[31]	*cTnI MHC-α >> MHC-β[29]MLC2-a >> MLC2-v*[30]*TTN-N2BA >> TTN-N2B*[31]
Gap junction localization	Random clustering, circumferential[18]	Co-localization with intercalated disks[18]
Metabolic		
Mitochondria morphology	Small, rounded, lacking cristae	Larger, elongated, with increased cristae surface area, 30% of cell volume[32,33]
Mitochondria localization	Nucleus or cell periphery[34]	Along myofibrils[34]
Method of energy production	Glycolysis (~80%)[26]	Fatty acid β-oxidation (~50–70%)[26]
Electrophysiologic/Functional		
Automaticity	Spontaneous	Beats only in response to stimuli
Resting membrane potential	−50 to 60 mV[31]	−70 to −80 mV[31]
Upstroke velocity	50 mV/ms[35]	150–300 mV/ms[35]
Action potential duration	Decreased	Lengthened
Calcium kinetics	Lowered Ca^{2+} release, influx velocity, and reuptake velocity from SR	Rapid Ca^{2+} release, influx velocity, and reuptake velocity from SR

iPSC-aCM: induced pluripotent stem cell-derived atrial cardiomyocytes; ssTnI: slow skeletal troponin I; cTnI: cardiac troponin I; MHC: myosin heavy chain; MLC: myosin light chain; TTN: titin.

Table 2. Comparison of mature atrial cardiomyocytes with immature and mature ventricular cardiomyocytes.

Mature atrial CMs	Immature ventricular CMs	Mature ventricular CMs
MHC-α (MYH6)[22]	MHC-α + MHC-β (MYH6 + MYH7)[22]	MHC-β (MYH7)[22]
MLC2-a (MYL7)[23]	MLC2-v + MLC2-a (MYL2 + MYL7)[23]	MLC2-v (MYL2)[23]
TTN-N2BA[24]	TTN-N2BA[24]	TTN-N2B[24]
CX43 + CX40 (GJA1 + GJA5)[22]	CX43 + CX40 (GJA1 + GJA5)[22,29]	CX43 (GJA1)[22,29]
KCNA5	–	–
KCNJ family, including KCNJ3	KCNJ family	KCNJ family, excluding KCNJ3
SLN[24]	–	–
NPPA	–	–

CM: cardiomyocytes; MHC: myosin heavy chain; MLC: myosin light chain; TTN: titin; CX: connexin.

commonly cited as the more immature isoform, but naturally matured atrial myocardium expresses a higher proportion of the N2BA isoform.[24]

Metabolic assessment

From heart development through terminal differentiation/formation and postnatal growth, cardiac metabolism undergoes dramatic change.[25] As a continually contracting organ, the heart has an extremely high energy demand and must supply itself constantly and efficiently with renewed ATP. The primary method of energy metabolism in early CMs is glycolysis utilizing glucose as the primary energy substrate, which promotes the proliferative (as opposed to hypertrophic) state of early CMs.[26,27] In natural CM development, once the CMs have terminally differentiated, the metabolic pathway switches to the more mature and efficient fatty acid *β*-oxidation.[28] Mitochondrial oxidative capacity is much more substantial and efficient than glycolytic metabolism, and thus aids in generating a more forceful and sustainable contractile function.[25] The mitochondrial and energy production factors that develop with aCM maturation translate to both an increased maximal oxygen consumption rate and mitochondrial respiratory capacity, as well as an upregulation in genes involved in fatty-acid *β*-oxidation, and downregulation of genes involved in lipid synthesis and glucose metabolism (including *CD36*, *CPT-1B*, *PDK4*, the peroxisome proliferator activated receptors—PPAR). Fatty acids also serve as ligands to increase phosphorylation, thus activating signaling pathways involved in translating external stimulation to internal responses, including ERK and p38 mitogen-activated protein kinase (MAPK).

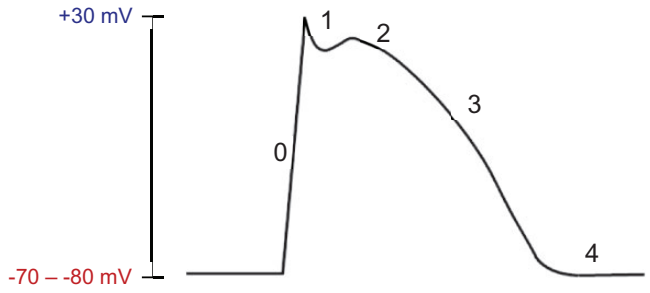
EP assessment

The heart is both electrical and mechanical, and each cardiac cycle and contraction is initiated by an electrical impulse that originates from pacemaker cells in the sino-atrial node. Due to cell–cell communication and the presence of non-parenchymal cells, this impulse is propagated to adjacent CMs, spreading throughout the heart. Each CM possesses an electrochemical gradient that relies on this electrical impulse. Once a CM receives the electrical impulse, the membrane potential is altered, which subsequently causes a cascade of activation/inactivation of membrane-bound and voltage-gated ion channels that mediate both the morphology and the magnitude of the AP. EP assessment relies on ion channel expression and function, with techniques such as single-cell whole-cell patch clamping (gold-standard), multielectrode arrays, and optical voltage imaging.

The cardiac AP consists of five phases: phase 0 (depolarization with rapid upstroke), phase 1 (transient repolarization), phase 2 (plateau), phase 3 (rapid repolarization), and phase 4 (resting membrane potential; Figure 2). The phases are mediated by the interplay of various ion channels (e.g. subtypes of Na^+, K^+, and Ca^{2+} channels). Factors that are crucial to developing a mature atrial cardiac AP include adequate expression and density of ion channels, appropriate localization of ion channels to structural and metabolic cellular machinery, and effective translation into functionality of the cell to generate a sufficient atrial AP. As with structural maturity, there are several EP characteristics that distinguish matured atrial CMs from ventricular CMs (Table 2). For example, gap junction/connexin expression differs between atria and ventricular myocardium. During fetal to adult transition in development, connexin 40 (CX40, *GJA5*) is expressed throughout both the atria and ventricles, but as maturation progresses, CX40 expression is restricted to the atria.[22] Connexin 43 (*GJA1*), on the other hand, is expressed throughout the myocardium in both atria and ventricles. The sole expression of CX43 in ventricles may explain the faster conduction velocity (CV) in ventricular myocardium.[29] There are also several genes that are solely present in atrial CMs, including *KCNA5*, *KCNJ3*, and *SLN*. *KCNA5* encodes for the ultrarapid delayed rectifier current I_{Kur}, and may provide an explanation for the shorter APD in atrial CMs compared with ventricular CMs. *KCNJ3* is unique to the atria, while other members of the KCNJ family are more highly expressed in the ventricular myocardium. Sarcolipin (*SLN*) is an atrial specific regulator of calcium handling and functions by inhibiting *SERCA2* via the lowering of *SERCA2a* affinity to calcium and thereby decreasing V_{max}.[24]

Current approaches and technologies to further mature iPSC-CMs

During embryonic development, CMs are exposed to environmental factors including extracellular matrix (ECM), electrical stimulation, mechanical signaling, soluble factors, and nutritional influx; these factors influence CM tissue architecture and function. Altering the physiochemical aspects of cellular microenvironment by mimicking



Phase	Current/Mechanism	Primary Genes
Phase 0: Depolarization, Rapid Upstroke	Na^+ Influx: I_{Na}	SCN5A
Phase 1: Transient Repolarization	Inactivation of Nav1.5	
	K^+ Efflux: I_{To} (transient outward)	Fast I_{To}: KCNA4, KCNA7, KCNC4 Slow I_{To}: KCND2, KCND3
	Ca^{2+} Influx: $I_{Ca,\ L}$	CACNA1C, CACNA1D
Phase 2: Plateau	Ca^{2+} influx: CICR	CACNA1C, CACNA1D, RYR2, CAMK2D, SERCA2, PLN, SLN
	K^+ efflux: I_{kur}	KCN5A
Phase 3: Repolarization	L-type Ca^{2+} channels dissipate K^+ influx:	
	I_{Kur}	KCN5A
	I_{Ks}	KCNQ1, KCNE1
	I_{Kr}	KCNH2, KCNE2
	I_{K1}	KCNJ3, KCNJs
Phase 4: Resting Membrane Potential	K^+ influx: I_{K1}	KCNJ3, KCNJs

Figure 2. Atrial action potential: the action potential is initiated by a depolarization (Phase 0) caused by a rapid influx of Na^+ ions, followed by a transient repolarization (Phase 1) mediated by an efflux of K^+ balanced with an influx of Ca^{2+}. This is followed by a plateau phase (Phase 2) maintained by massive influx of Ca^{2+} through calcium-induced calcium release from the sarcoplasmic reticulum, and efflux of K^+ through the quickly activating but slower inactivating ultrarapid delayed rectifier, followed by a late repolarization phase (Phase 3) primarily induced by dissipation of Ca^{2+} and K^+ efflux through activation of several K^+ channels, before returning to the resting state (Phase 4). (A color version of this figure is available in the online journal.)

physiological mechanical load, electrical pacing, 3D culture, supplementing with soluble factors, and/or engineering ECM geometry and modulating ECM substrate stiffness are some strategies for directing iPSC-CMs towards adult-like structural and functional maturation (Figure 3).[12,13,20,30,31] However, currently even the most advanced methods of maturation result in a lack of maturity that fails to match even the late fetal stages of CM maturity (Figure 4). Understanding natural fetal development would help to bridge our understanding of progression of iPSC-CMs to the adult mature phenotype.[32] Most maturation approaches have focused on ventricular CMs or a heterogeneous population of nodal, ventricular, and atrial cells. Less is known about the effects of these maturation approaches on iPSC-aCMs, a necessity to faithfully model AF in a dish.

Postnatal, biochemical, and metabolic conditioning

Postnatal and biochemical conditioning that have been investigated on iPSC-derived ventricular CMs include triiodothyronine (T3), insulin-like growth factor-1 (IGF-1), and dexamethasone.[33–35] Towards the end of gestation, fetal cortisol concentrations rise prepartum in preparation for extrauterine survival. The preparation for the postpartum life is dependent on a prepartum rise in fetal serum cortisol concentration, which activates adaptations in cardiac function.[34] Rise in plasma T3, a primary driver in the maturation of fetal CMs in humans, primarily occurs in the final 10 weeks of gestation and when T3 levels are severely reduced, several characteristics of CM growth and maturation are negatively affected.[36] T3 also acts indirectly by controlling the bioavailability and efficacy of other hormones crucial to fetal development and growth, including IGF-1, which is important in fetal and placental growth. T3, IGF-1, and dexamethasone (collective known as "TID") provide maturational signals that are especially important for developing binucleated CMs and cardiac contractile proteins in human fetuses, and improve cell size and resting membrane potential.[33] Postnatal and biochemical conditioning likely also has a positive effect on the metabolic maturity of cells, such as increased ATP production, increased expression of *PGC-1α* and *PGC-1β* (fatty acid oxidation regulators), enhanced mitochondrial function, and decreased levels of reactive oxygen species.[33]

The nutritional substrate that the developing heart receives plays a crucial role in determining the metabolic

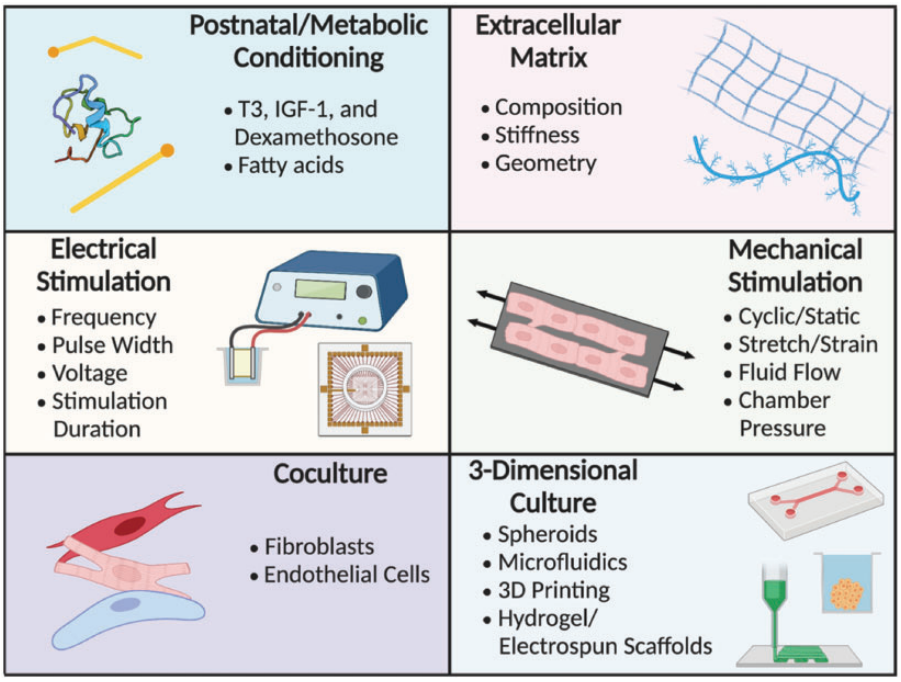


Figure 3. Methods for iPSC-derived atrial cardiomyocyte maturation: postnatal and metabolic conditioning, extracellular matrix, electrical stimulation, mechanical stimulation, co-culture, and three-dimensional culture have all been utilized to increase iPSC-derived cardiomyocyte maturity in culture. Figure created using BioRender.com. (A color version of this figure is available in the online journal.)

maturation of CMs. Fetal cardiac glucose uptake is drastically reduced in the late fetal stage to prime CMs for the drastic shift from placental nutrition to breast milk containing various fatty acids.[37,38] As CMs reach terminal differentiation, metabolism shifts to increased reliance on mitochondrial β-oxidation to more efficiently generate ATP, precluding the CMs' ability to metabolize the diverse carbon sources that the native myocardium receives from blood.[39] High glucose suppresses cardiac maturation, increases mitotic activity, and causes the myocardium to be more susceptible to congenital heart disease.[40] Typical iPSC-CM media is glucose rich and lipid poor, which promotes lipogenesis and suppresses fatty acid oxidation. Fatty acid supplementation focuses on optimizing metabolic maturation of iPSC-aCMs by increasing their dependence on fatty acids, decreasing basal glycolytic activity, and improving adaptability to environmental changes.[41] Encouraging cells to rely on fatty acid metabolism by limiting their access to glucose can increase/enhance (a) transcription of genes related to fatty acid metabolism, (b) sarcomeric length and expression of mature structural markers, (c) upstroke velocity and AP duration at 90% (APD90) and cardiac-specific Na^+, K^+, and Ca^{2+} handling genes, and (d) force production and calcium dynamics.[41–43]

ECM modulation

The ECM, with its organ-specific composition, stiffness, and geometry, plays an important role in stem cell fate decisions, normal development, and cardiogenesis.[12] Methods for modulating ECM composition include the use of individual and combined ECM proteins that comprise the native myocardium, including collagen I/III (structural support and organization), collagen IV (basement membrane formation and cellular alignment facilitation), fibronectin (integrin connection to other ECM proteins and migration of cardiac precursor cells), and laminin (sarcomeric organization).[32,44] The use of cardiac decellularized ECM (dECM) has demonstrated promising results due to its high bioactivity, ability to revascularize, and use as a biological scaffold for cardiac regeneration and prevention of fibrosis; fetal dECM, in particular, can provide regenerative and proliferative signals involved in cardiac development.[32,45]

The stiffness of ECM can also modulate gene expression, producing more robust and highly organized gap junctions as compared with stiff surfaces. Specifically, culture on softer ECM improves CV, increases K^+ and Na^+ current densities, increases the expression and localization of intercellular gap junction proteins, induces hypertrophic and mature isoforms thereby promoting greater tension development and responsiveness to autonomic input, and increases integrin expression and activation.[12,46] Furthermore, dynamically increasing ECM stiffness over time significantly upregulates AKT and p38 MAPK mechanosensitive pathways.[32]

ECM patterning helps control the geometry for cell adhesion, and iPSC-CMs show improved sarcomere formation, cell alignment, transverse tubule development, Ca^{2+} handling, and CV on patterned ECM.[47] Such alignment enhances focal adhesion formation, improves polarization from cell elongation, and controls directionality of cell–cell junctions, thus also impacting sarcomeric organization, contraction-induced mechanical loading, and AP propagation.[48] Micropatterning also improves cell–cell contacts towards replicating the polarized fibrous tissue structures

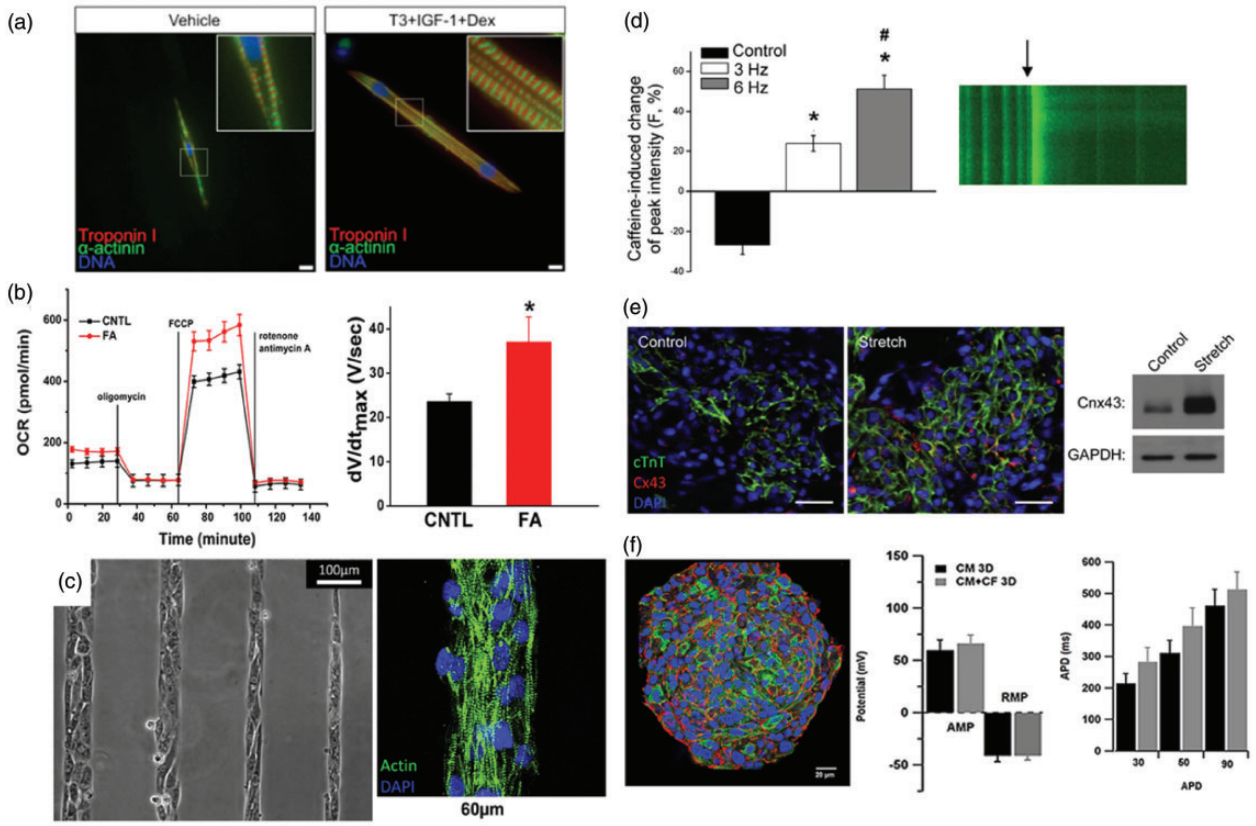


Figure 4. Bioengineered models of cardiomyocytes and cardiac tissue: (a) Treatment of iPSC-CMs with postnatal factors triiodothyronine (T3), insulin-like growth factor-1 (IGF-1), and dexamethasone (Dex) leads to increased cardiomyocyte size and sarcomere alignment. Figure adapted from Birket *et al*.[33] (b) Representative traces for control and fatty acid (FA)-treated hPSC-CMs responding to the ATP synthase inhibitor oligomycin, the respiratory uncoupler FCCP, and the respiratory chain blockers, rotenone and antimycin A. Higher maximal oxygen consumption rate (OCR) was seen in FA-treated versus control (CNTL) cells. Upstroke velocity (right) was also increased with FA treatment. Figure adapted from Yang *et al*.[37] (c) Micropatterned cardiomyocytes led to alignment of cardiomyocytes and their actin filaments. Figure adapted from Salick *et al*.[48] (d) Electrical stimulation promoted improvement in Ca^{2+} handling properties as evident by non-stimulated control cells not responding to caffeine while stimulated cells responded to caffeine by releasing more calcium ions. Fluorescence recording (right) of calcium transients before and after administration of caffeine (arrow) in cells exposed to 6-Hz electrical stimulation. Figure adapted from Sun and Nunes.[20] (e) Mechanical stimulation of iPSC-CMs led to an increase in Connexin 43 (Cx43 and Cnx43) formation compared with static controls. Figure adapted from Mihic *et al*.[57] (f) Three-dimensional culture (N-cadherin (red) and EH-myomesin (green)) as well as co-culture with cardiac fibroblasts (CF) lead to increases in APD and amplitude (AMP). Figure adapted from Beauchamp *et al*.[71] (A color version of this figure is available in the online journal.)

of CMs.[48,49] CMs form intercalated disks with neighboring cells axially and form costameres with ECM ligands laterally.[48]

In vitro mechanical and electrical stimulation of iPSC-CMs

Electrical signals and mechanical loading are major determinants of *in vivo* CM development/maturation to prime the CMs and the native myocardium to appropriately respond to electrical conduction and constant contraction. Electrical signals are known to play an important role during fetal development, and *in vivo*, direct current electric fields are involved in embryonic development, the disruption of which leads to abnormal development.[50] External electrical stimulation has the potential to mimic the electrical stimuli to which the native heart responds. Electrical stimulation greatly improves ventricular phenotype and maturity by targeting cell volume/size, sarcomeric banding and alignment, and myofilament ultrastructure with increased expression of adult atrial isoforms of myosin light and heavy chains, and cardiac troponins.[31,50,51] Improvement with sarcomeric organization and structure is accompanied by enhanced contractile force and tensile stiffness, as well as oxidative metabolism and energetics with enhanced transport of nutrients and metabolites during contraction.[20,52,53] Electrical stimulation also directly impacts ion channels and key Ca^{2+} handling pathways, particularly via localization of T-tubules to cardiac calcium pump (*SERCA2*) and sodium-calcium exchanger (*NCX*), expression of Ca^{2+}-induced calcium release modulators (*RYR2*), other Ca^{2+} handling channels and regulators, improved gap junction formation, and decreased automaticity, as well as correlated improvement in cardiac gene expression.[20,31,51,52,54,55]

Electrical stimulation has been explored alone, as seen with 2D monolayers, cardiac biowires,[56] and tissue constructs, or in combination with different types of mechanical stress such as fluid flow, cyclic strain, or static stress. Mechanical stimulation is characterized by systems that physiologically mimic blood flow and cardiac cycles by using fluid flow, chamber pressure, and cyclic or static strain. These systems have been shown to improve

functionality and contractility, with increases in *SERCA2*, L-type Ca^{2+} channels, and RyR expression, as well as an increase in electrical coupling. Mechanical stimulation also enhances structural maturity with an increase in cTnT, MHC-β and connexin-43 (*GJA1* ventricular specific; atrial isoforms include *MHC-α* and *GJA5*), and improved sarcomeric organization and Z-disk formation.[57] Mechanical stimulation also improves the expression of *KCNJ2*, one of the K^+ channels responsible for resting membrane potential maintenance.[57–59] When electrical stimulation is combined with static stress, an increase in *SERCA2* and hypertrophy is observed, and when combined with cyclic/static stress, SERCA2 and RYR2 increase, along with improved sarcomeric alignment, contractility, electrical coupling, and CV.[31] Determining if electromechanical stimulation alone or in combination with other maturation approaches enhances iPSC-aCM maturity is important as genetic variants in *TTN,* the gene encoding the sarcomeric protein titin, are not only associated with dilated cardiomyopathy, but also AF.

Co-culture of iPSC-CMs with fibroblasts and endothelial cells

Cardiac maturation/functionality is orchestrated via cross-talk with various cell types. Although CMs maintain the largest cell occupancy by volume,[60] CMs constitute 20–30% of the total numbers of the cells in the heart, while endothelial cells comprise ~25% and cardiac fibroblasts (CFs) comprise ~45–55%.[61] CFs are in direct contact with CMs[61,62] and interact via paracrine signaling, cell surface molecules, and ECM interactions.[63] CFs also express voltage-dependent Na^+ channels which allow for inward current. Though not inherently excitable, CFs can modulate CM EP properties.[64] In addition, gap junctions, specifically *GJA1* and *GJA5*,[65,66] allow for impulses to be directly passed between cells and activate electromechanical transduction pathways. CM/CF ratio modulation influences the electrical characteristics of the CMs, and by inhibiting *GJA1* expression, cell–cell gap junction communication modulates electrical functionality.[67] The balance between ECM production and degradation by CFs is finely tuned, perturbation of which can cause pathologic remodeling via CF differentiation to myofibroblasts.[68–70]

Co-culture of iPSC-CMs with CFs in 3D spheroids leads to increased AP magnitude and duration compared with 2D controls.[71] Combining the iPSC-CM/CF co-culture with a collagen hydrogel exposed to mechanical stimulation synergistically allowed for improved ECM remodeling and cell alignment as well as decreased (more mature) beating rate.[72] In another study, encapsulation of iPSC-CMs and fibroblasts (dermal) within poly(ethylene glycol) hydrogels followed by layer-by-layer coating/deposition of fibronectin and gelatin onto the hydrogels improved cell–cell interactions, viability, and increased the beating strength two-fold as compared with iPSC-CMs in hydrogels without any ECM coating.[73] Lastly, fetal CFs induced a higher expression of functional cardiac genes while adult CFs led to fibrotic-like state with decreased CV, prolonged APD, and decreased Ca^{2+} transient amplitude.[74]

Cardiac endothelial cells (CEs) provide a necessary barrier that acts as a gateway between the myocardium and the blood by lining the endocardium and blood vessels, and the high-density capillary networks that form throughout the myocardium in close contact with CMs help meet the metabolic needs of the cardiac tissue.[75,76] During development, CEs produce myocardial maturation signals, including platelet-derived growth factor and neuregulin, which binds to the epidermal growth factor receptor family on CMs leading to proliferation, survival, and hypertrophy of neonatal CMs.[77–79] In the adult heart, CEs regulate contractility through secretion of nitric oxide (NO) and endothelian-1[80] and regulate hypertrophy, oxidative stress, and inflammation through factors including angiotensin II, prostaglandin I_2, endothelin-1, and NO.[81–83] CEs also improve cellular alignment and activate numerous developmental pathways in iPSC-CMs including Ca^{2+} handling and sarcomeric structural genes[82] in 2D monolayers, 3D hydrogels, hanging drop cultures, and spheroids.[84–87] Because CEs are exposed to shear stress due to blood flow, microfluidic devices are increasingly utilized to study EC biology and CM-CE interaction. In dynamic microfluidic devices that capture the effects of blood flow, the physiologic sheer stress can lead to phenotypic improvements in the ECs, including cellular alignment.[88]

Three-dimensional culture of iPSC-CMs in different platforms

The 2D culture platforms are unable to model the native 3D heart architecture, which comprises of uniaxially compacted ECM and cardiac cells.[35,89–92] The simplest approach to 3D cardiac models is formation of spheroids via self-clustering of cells on a non-fouling culture substrate (e.g. non-adhesive culturing dish, hanging drop devices).[71,82,93–99] Compared with 2D culture, 3D spheroidal cultivation accelerates the structural maturation of iPSC-CMs with upregulated expression of cTnT, sarcomeric α-actinin, and sarcomere length.[98] The 3D spheroids also improve metabolic maturation at both the molecular and fluxome levels with downregulated glycolysis and lipid biosynthesis, and upregulated oxidative phosphorylation.[97] Spheroid culture can be easily integrated into other maturation techniques such as co-culture and electrical stimulation.[82,99] However, the spheroid model for iPSC-CMs has limitations, including phenotype modulation from atrial to ventricular, poor cell-ECM signaling-mediated maturation, lack of anisotropic alignment of CMs, and unidirectional mechanical stretch.[94,95,97,98]

Electrospinning technology that produces anisotropic fibrous scaffolds incorporates ECM modulation with alignment.[100,101] Anisotropically electrospinning biocompatible polymers such as poly-ε-caprolactone (PCL) and polyurethane result in efficient construction of a highly ordered fibrous scaffold exhibiting a high and tunable surface-to-volume ratio, which allows anisotropic alignment of iPSC-CMs in a direction that is parallel to the fibers.[100–103] In contrast to culture on bare tissue culture polystyrene, iPSCs showed more elongated cellular morphology, increased expression of genes encoding for structural

proteins (e.g. *TNNT2, MYL7,* and *TTN*), improved sarcomeric organization, and improvements in ion channels/Ca^{2+} handling on electrospun aligned scaffolds.[104] However, iPSC-CM penetration into electrospun scaffolds is generally limited, which does not fully mimic the native human myocardium.[104]

The utilization of a soft 3D ECM hydrogel that exhibits lower stiffness than PCL fibers as a cell-anchoring matrix has been extensively studied.[35,95,105,106] Although the inherent polymeric structure of ECM hydrogels does not allow for uniaxiality and static tension, anisotropic ECM orientation can be generated by applying supportive structures such as post, pillars, pins, and clips during the hydrogel forming process, all of which can be engineered to also induce mechanical tension and synchronized contraction/stretching of the aligned iPSC-CMs.[107–111] The auxotonic contraction of CMs encapsulated in a hydrogel can be manipulated by adjusting the mechanical property of supportive structures.

Despite improving CM maturation and enabling electromechanical stimulation, hydrogel-based 3D cardiac platforms pose difficulty in scaling up the study throughput due to complex and expensive fabrication/operation of support structures and mechanical tools.[112–114] On the other hand, 3D printing technology (e.g. microextrusion method, ink-jet method, and stereolithography) has the potential to overcome this barrier with large/macro scale tissue generation of hydrogel-based 3D cardiac structures.[115,116] For example, gelatin methacrylate and alginate-based microfibrous scaffold with encapsulated endothelial cells was 3D printed using a microextrusion-based bioprinter to form a vascular bed, which was then seeded with rat neonatal CM and human iPSC-CMs, resulting in improved sarcomere structure and contractility in the anisotropic scaffold exhibiting higher aspect ratios of microfibrous structures compared with the isotropic scaffold.[117] Although 3D printing is highly promising in cardiac tissue engineering for drug screening and maturation platforms, more comprehensive studies are still required to demonstrate maturation parameters and application utility relative to simpler model systems.

Conclusions and future outlook

Generation of patient-derived iPSC-aCMs has great potential to aid in the development of novel therapies that target the underlying abnormalities in AF-causing mutations. However, while large strides have been made to further mature iPSC-aCMs, they still do not fully recapitulate the full mature phenotype of adult aCMs. Furthermore, there is currently no universal metric(s) for defining when an iPSC-aCM has met acceptable maturation for the desired endpoint application. Continued research is required to determine key processes in fetal cardiac development that synergistically lead to aCM maturation. A balance of adapting natural developmental cues and mimicking the developing microenvironment with the incorporation of advanced engineering techniques such as those discussed above can likely improve iPSC-aCM maturation more efficiently in high-throughput platforms for specific applications.

The purity of differentiated iPSC-aCMs must also be improved. Protocols to generate iPSC-aCMs are less efficient than those for the ventricular phenotype[118] or require suspension cultures based on embryoid body formation.[16] Large pharmaceutical screens require high yields of uniform populations of iPSC-aCMs that can be readily reproduced; therefore, advances in differentiating purer iPSC-aCM populations as well as reducing batch-to-batch variability will be vital for pharmaceutical screening of novel AF therapies. Availability and use of technologies such as glucose starvation,[119] antibiotic resistant cassettes,[120] and cell sorting strategies such as magnetic bead sorting[121] to select for specific iPSC-aCM populations will reduce experimental variability. Lastly, the ability to derive aCMs, endothelial cells, and fibroblasts from the same iPSC lines will aid in the fabrication of fully personalized AF platforms.

In conclusion, engineered heart models for the study of AF are being fabricated with varying cellular and technological complexities based on the specific application. Further maturation of iPSC-aCMs using advanced engineering techniques will lead to improved patient-specific evaluation of the underlying causes of AF. Such an outcome will undoubtedly improve treatment options for patients and lead to a better understanding of heart disease.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, literature review, writing, and revision process of the manuscript: OTL, GEB, YDH, DD, SRK, OTL, GEB, and YDH wrote the manuscript, and DD and SRK provided feedback and revised the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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ORCID iD

Olivia T Ly https://orcid.org/0000-0002-9658-9643

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