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Engineering hiPSC cardiomyocyte *in vitro* model systems for functional and structural assessment

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Abstract

The study of human cardiomyopathies and the development and testing of new therapies has long been limited by the availability of appropriate *in vitro* model systems. Cardiomyocytes are highly specialized cells whose internal structure and contractile function are sensitive to the local microenvironment and the combination of mechanical and biochemical cues they receive. The complementary technologies of human induced pluripotent stem cell (hiPSC) derived cardiomyocytes (CMs) and microphysiological systems (MPS) allow for precise control of the genetics and microenvironment of human cells in *in vitro* contexts. These combined systems also enable quantitative measurement of mechanical function and intracellular organization. This review describes relevant factors in the myocardium microenvironment that affect CM structure and mechanical function and demonstrates the application of several engineered microphysiological systems for studying development, disease, and drug discovery.

Keywords

In vitro cardiac model; Human induced pluripotent stem cell; derived cardiomyocytes (hiPSC-CMs); Microphysiological systems (MPS); Heart-on-a-chip; Cardiac mechanobiology; Drug discovery

1. Introduction

Cardiovascular disease is the leading cause of death in the developed world. A large fraction of the morbidity and mortality is the result of cardiomyopathies that affect cardiomyocytes (CMs: specialized muscle cells of the heart) (Benjamin et al., 2018). CMs are arranged in vascularized anisotropic layers within the extra-cellular matrix (ECM) structure of the heart to allow for coordinated organ pumping (Riegler et al., 2015; Greenbaum et al., 1981; Arts et al., 2001). Sarcomeres, the fundamental contractile unit of cardiomyocytes are connected in series into cell-spanning myofibrils, which are anchored to the cells' microenvironment (including ECM and neighboring cells) through specialized cellular adhesion complexes (Pardo et al., 1983; Clark et al., 2002). Subtle disruption of the internal structures within

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cardiomyocytes or the organization and adhesion of cells within their ECM scaffold can have profound effects on cellular function and cytoskeletal organization, which in turn leads to organ level remodeling and disease (Ferreira–Cornwell et al., 2002; Fatkin et al., 2014; Spudich, 2014; Davis et al., 2016).

Historically, animal models have been the primary system used to study cardiovascular diseases at the organ and organism level. Large animal models (pigs, sheep, monkeys) can provide a close match for the hemodynamic environment and protein expression found in humans, but their use in research is limited because of the cost to house and maintain them, as well as the difficulty of creating genetic models (Dixon and Spinale, 2009; Milani–Nejad and Janssen, 2014). Small rodents (mice and rats) have been used more extensively because of their fast life cycle and genetic tractability, but these animals are not ideal models for human physiology (Povsic et al., 2017; Camacho et al., 2016). Outside of the obvious differences in hemodynamics (rats ~350; mice ~650; humans 60 beats per min), variation in the expression of proteins that generate force and control the electrical activity in the heart have limited the accuracy of small animal models for disease studies and drug development (Denayer et al., 2014; Milani–Nejad and Janssen, 2014). For instance, beta cardiac myosin accounts for 95% of the sarcomeric myosin in mature human ventricular myocytes, and is the site of roughly 35% of the known mutations which cause hypertrophic cardiomyopathy, but is only expressed during development and disease in small rodent hearts (Weiss and Leinwand, 1996; Spudich, 2014). Several proposed and developing therapies for heart failure and cardiomyopathies (including omecamtiv mercabil) have been aimed at altering the kinetics of myosin motor function, but the known differences in myosin kinetics between species may limit the predictive power of mouse models (Planelles–Herrero et al., 2017; Spudich, 2014; Tardiff et al., 2015; Nagy et al., 2015; Malik et al., 2011). Furthermore, differences in ion channel expression (notably hERG channels) can complicate modeling long QT syndrome and the effect of anti–arrhythmic drugs that target these channels (Spencer et al., 2014; Salama and London, 2007; Sanguinetti and Mitcheson, 2005). Cardiotoxicity induced by cancer therapies including doxorubicin and sunitinib is variable in humans, and potential genetic factors that contribute to this heterogeneity are difficult to study in mouse models (Force and Kolaja, 2011; Cheng and Force, 2010; Kerkelä et al., 2006; Chu et al., 2007; Burke et al., 2016; Arad et al., 2002). Finally, the complexity of the *in vivo* environment – dynamically remodeling tissue properties (Weber et al., 1988), multicellular components (Pinto et al., 2016), and chemical signals from around the body (Ammarguella et al., 2002; Ross, 2004) – complicates the measurement and interpretation of specific CM phenotypes.

A human–derived cell model system for studying cardiomyocyte function and dysfunction could address some of the shortcomings of animal models, but cell sources are limited (Pluess and Ehler, 2015). Primary human cardiomyocytes are difficult to obtain, maintain in culture, and manipulate using modern molecular biology tools like CRISPR/Cas9 mediated gene editing (Parameswaran et al., 2013). This limits their usefulness for studying disease mechanisms or adaptive cellular responses. There have been some studies using excised cardiac tissues from myectomy samples that can be cultured for up to 4 weeks, but viable human myectomy samples are quite scarce (Brandenburger et al., 2012; Kang et al., 2016). Embryonic or mesenchymal stem cell derived cardiomyocytes provided an alternative,

scalable cell source, but acquisition and differentiation of these cells has historically been relatively expensive and inefficient (Laflamme et al., 2007; Fernandes et al., 2010; Rangappa et al., 2003).

Human induced pluripotent stem cell (hiPSC) derived CMs have gained traction as a scalable cell source derived from patients (or edited to match specific human mutations) (Zhang et al., 2009). These cells are a powerful tool for human disease modeling and drug screening with the potential to enable personalized precision medicine (Gowran et al., 2016; Sayed et al., 2016). The establishment of increasingly robust and cost-effective protocols for differentiating these cells into cardiomyocyte-like cells has greatly increased their use (Yang et al., 2014; Chen et al., 2015b; Burridge et al., 2015; Lian et al., 2013). More recent studies of human embryonic stem cells have similarly improved the efficiency of directed differentiation into cardiomyocytes and shown very similar molecular characterization to hiPSCs, but the acquisition and use of human embryonic stem cell lines has more regulatory barriers than hiPSCs (Mallon et al., 2014; Kobold et al., 2015). Many studies have reported observable phenotypes, including less robust or organized myofibrils, in hiPSC-CMs with mutations linked to human disease (Lan et al., 2013; Wang et al., 2014; Karakikes et al., 2014; Hinson et al., 2015; Gowran et al., 2016; Chopra et al., 2018). Some studies have used patch clamping and multielectrode arrays to study the effects of mutations in ion channels on iPSC-CM electrophysiology (Braam et al., 2013; Sala et al., 2016; Bellin et al., 2013; Davis et al., 2012). Several of mutations linked to cardiomyopathies also disrupt the calcium handling process, which can be visualized with live cell imaging of cells in 2D culture (Lan et al., 2013; Sun et al., 2012; Wyles et al., 2016). Live-cell imaging techniques have also been used to observe intracellular movement generated by the contractile structures within iPSC-CMs, which is affected by some disease causing mutations (Laurila et al., 2016; Lan et al., 2013; Sun et al., 2012). However, the constraint of adherence to a rigid substrate means that these measurements cannot accurately capture the forces generated by the cells or replicate fundamental responses to length change and pressure. The usefulness of these cells for studies of structure and mechanical function has traditionally been limited by cell population heterogeneity, immaturity, and a stiff 2D culture environment.

Currently, many hiPSCs are grown and differentiated on tissue culture plastic with physisorbed matrix proteins such as laminin, fibronectin, or Matrigel (Lam and Longaker, 2012; Kohen et al., 2009; Hughes et al., 2010). Tissue culture plastic has a stiffness of around 3 GPa, nearly a million times stiffer than the native environment of the myocardium (Acevedo-Acevedo et al., 2015). Additionally, whether the proteins/growth factors are physisorbed or chemisorbed to the substrate may change bioactivity of the proteins (Psarra et al., 2015). This differentiation protocol results in a spontaneously beating, multilayered sheet of cells characterized by high heterogeneity both within each batch, between different batches, and between different labs. Some groups have demonstrated that culturing the cells in a 3D spheroid culture or 3D adhered culture improves some functional measures of maturity (Kerscher et al., 2016; Nguyen et al., 2014). Others have shown that implanting iPSC-derived cells into a living heart greatly improves maturation (Kadota et al., 2017). Mimicking elements of the native microenvironment may be a key step to expand the utility of hiPSC-CMs for studying human cardiomyocyte structure and function *in vitro*.

Despite the potential of iPSC-CMs, immature, embryonic-like phenotype and large intrapopulation heterogeneity limit their ability to model adult cardiomyocyte physiology (Karakikes et al., 2015). Some of the key metrics of immaturity are the features of the contractile structures, myofibrils, which are less aligned and less densely packed in hiPSC-CMs compared to native CMs (Yang et al., 2014; Ruan et al., 2016). This lack of maturity is also reflected in the ion channel expression, calcium handling, t-tubule organization, and resultant electrophysiology of these cells (Ronaldson-Bouchard et al., 2018; Denning et al., 2016). Gene expression and protein analysis of receptors and sarcomeric proteins indicate that iPSC-CMs resemble early embryonic CMs and are lacking several important functional proteins expressed in mature human ventricular myocardium (eg. MYH7, TNNI3, and ADRB1) (Gowran et al., 2016; Leonard et al., 2018; Ronaldson-Bouchard et al., 2018). Modeling of specific cardiac cell responses with stem-cell derived cardiomyocytes has been limited by the absence of certain receptors (eg. Beta adrenergic receptor 1) until a more mature state is reached (Jung et al., 2016). Strategies of varying complexity have been proposed to increase the maturity of cells, including temporal maturation windows, specific media compositions, electrical pacing, cyclic stretch (Tandon et al., 2009; Yang et al., 2014; Richards et al., 2016). Many of these strategies aim to direct the cells down a mature lineage by shifting their metabolism and cell cycle state via addition of media components (Wheelwright et al., 2018; Correia et al., 2017). The lack of maturity in hiPSC-CMs has also been speculated to be due in part to the different environmental cues they receive in 2D cell culture compared to their native environment *in vivo* (Eschenhagen et al., 2015).

In recent years, different types of microphysiological systems (MPS) have been engineered to provide an *in vitro* environment more similar to the native *in vivo* environment, to improve the ability of hiPSC-CMs to match that of native, mature CMs (Fig. 1). MPS utilize bioengineering and biology to model native tissue functions *in vitro* (DARPA/DSO, 2011). Substrate stiffness, particular ECM availability, and interactions with neighboring cells have all been tuned in MPS and have been shown to modulate cell phenotypes, protein expression, and cytoskeletal organization (Tzatzalos et al., 2016; Mathur et al., 2015; Huebsch et al., 2016; Ronaldson-Bouchard et al., 2018; Ulmer et al., 2018). Additional controllable inputs, including specialized media components and electrical pacing (at both physiologic rates of 1 Hz and extraphysiologic rates of 6–10 Hz) have also been shown to promote maturity in these cell populations (Nunes et al., 2013; Hirt et al., 2014; Ronaldson-Bouchard et al., 2018; Mills et al., 2017). While the structural and functional maturity of the stem-cell derived cells in these constructs falls short of native cells in excised tissue, they are much improved over traditional hiPSC-CMs. Furthermore, many of these platforms are designed to specifically enable measurement of force generation by cardiomyocytes in more physiologically relevant mechanical contexts (Laurila et al., 2016; McCain et al., 2013; Sidorov et al., 2017). Some of these systems focus on single cells to allow for detailed observation of remodeling of intracellular structures including myofibrils, sarcomeres and adhesion complexes (Ribeiro et al., 2015; Aratyn-Schaus et al., 2016; Chopra et al., 2018). Single cell approaches also attempt to address the high degree of heterogeneity in the population of cells acquired during the differentiation process (Ribeiro et al., 2017). Other systems use multicellular constructs to form microtissues, allowing the observation of tissue level functions by incorporating more native-like cell-cell adhesion, but making the

deconvolution of differences in heterogeneous cell populations more challenging (Sidorov et al., 2017; Ronaldson–Bouchard et al., 2018; Mathur et al., 2015; Mills et al., 2017).

While certain questions about cardiac function are testable in hiPSC–CMs grown in traditional 2D formats, MPS are better *in vitro* platforms to study structural and functional phenotypes of cardiac disease and drug responses because of two key features. First, MPS promote a more mature hiPSC–CM morphology (e.g. aligned sarcomere, pronounced T–tubules) and function (e.g. lower resting membrane potential, expression of adult CM proteins, contraction) by recapitulating aspects of the cardiac microenvironment. Second, many of these platforms are designed to allow for quantitative, physiologically relevant measurement of mechanical output. In the following sections, we describe the native environment of the heart and cardiac MPS which use hiPSC–CMs to model human cardiac function and disease and support drug discovery.

2. Microenvironment of the native heart

The microenvironment of CMs consists of various biophysical and biochemical cues that guide CM behavior and function (Fig. 2). Cells (including cardiomyocytes) can sense changes in their local microenvironment through mechanosensitive adhesion proteins and their associated protein complexes (Jacot et al., 2010; Majkut et al., 2014). These adhesion complexes form a mechanical link between the exterior and interior structures of cells and transmit intracellular signals to initiate changes in gene transcription and cellular morphology and function (Samarel, 2005; Pluess and Ehler, 2015).

2.1. Multicellular structure

The heart wall is composed of CMs, endocardial cells, epicardial cells, cardiac fibroblasts, cardiac endothelial cells, leukocytes and macrophages (Rienks et al., 2014; Zhou and Pu, 2016). CMs comprise the largest fraction of the volume of the heart and are responsible for the contraction of the heart (Zak, 1973; Bayomy et al., 2012). Distinct subtypes including atrial and ventricular CMs and cardiac conduction cells are distributed throughout the different chambers of the heart and have unique functional characteristics including protein expression and conduction profiles (Später et al., 2014). This specialization and spatial arrangement enables coordinated contraction roughly once a second, generating strains ranging from -0.2 to 0.4 and tensile and compressive stresses throughout the myocardium (Huang et al., 2017b). The propagation of contraction through the tissue is mediated by mechanical and electrical cell–cell contacts with other cardiomyocytes and cardiac fibroblasts (Vasquez et al., 2011; Kohl and Gourdie, 2014). Cardiac fibroblasts are responsible for secreting and maintaining the ECM scaffold, a major determinant of the heart's mechanical properties (Bayomy et al., 2012; Schroer and Merryman, 2015). Both CMs and cardiac fibroblasts are sensitive to cyclic stress and strain, which can activate intracellular signaling and remodeling (Jacot et al., 2010; Schroer and Merryman, 2015). Cardiac endothelial cells form the vasculature of the heart and influence cardiac morphogenesis and homeostasis by releasing paracrine signaling factors (Zhou and Pu, 2016; Brutsaert, 2003; Pinto et al., 2016). The vasculature allows for nutrient exchange and transport of soluble signaling factors and immune cells to the heart, which can have major

effects on the development and remodeling after disease (Epelman et al., 2015; Ma et al., 2018). Recent studies have highlighted the roles of resident macrophages and recruited leukocytes in the maintenance of homeostasis and injury response (Chen and Frangogiannis, 2016; Sager et al., 2016). Finally, epicardial and endocardial cell layers on the external and internal surfaces of the heart play key roles in the heart's development and regeneration, as they contribute to paracrine signaling and give rise to the progenitors of cardiac fibroblasts and endothelial cells in the myocardium (Masters and Riley, 2014; Luxàn et al., 2016; Haack and Abdelilah-Seyfried, 2016).

2.2. Mechanical properties

Biophysical properties including structural features, mechanical properties and electrical conductivity have significant effects on cardiac cell function (Atmanli et al., 2017). In healthy myocardial tissue, cells exist in an aligned (and therefore anisotropic) structure with cardiac fibroblasts maintaining ECM homeostasis and blood vessels interspersed throughout. The combination of cells and ECM contribute to bulk mechanical properties including elastic modulus and viscoelasticity (Wang et al., 2016). A variety of techniques, including assessment of echocardiogram/MRI imaging of contracting hearts, bulk tissue mechanical measurements, and microscale atomic force microscopy have all been used to measure the mechanical properties of myocardial tissue (Yao et al., 2012; Pislaru et al., 2014). Viscoelasticity has been measured based on high-frequency echocardiograms and is affected by cardiac conditions including myocardial infarction (Pislaru et al., 2014). The normal stiffness (elastic modulus, E) of the embryonic myocardium has been reported to be around 10 kPa. (Engler et al., 2008). Furthermore, embryonic and neonatal CMs cultured on hydrogels with a stiffness around 10 kPa have shown improved contractile function compared with their function in softer or stiffer environments (Engler et al., 2008; Bhana et al., 2010).

2.3. Organized ECM

Bulk tissue mechanical properties depend on the intrinsic material properties of ECM proteins and cells as well as the arrangement and linkages within these structures. The ECM is comprised of a variety of fibrillar proteins aligned circumferentially and anisotropically around the chambers (Greenbaum et al., 1981; Hanson et al., 2013). In healthy myocardium the ECM fibers orientation is longitudinally along CMs with few crosslinks (Bayomy et al., 2012). Collagen I is the most abundant ECM protein in the heart and together with Collagen III maintain structural integrity of the heart (Thimm et al., 2015; Singelyn and Christman, 2011). Collagen I provides tensile strength while collagen III provides distensibility to the structure of myocardium (Takawale et al., 2015; Segura et al., 2014). Basement membrane occupies the interstitial space between ECM and CMs and includes proteins such as laminin, fibrillin, fibronectin and collagen type IV, as well as proteoglycans which can bind soluble growth factors and cytokines (Takawale et al., 2015). In addition to their contribution to the mechanical properties of the myocardium, the specific composition of the ECM can trigger activation of biochemical signaling through the binding of specific integrin types to cell adhesion ligands found in many ECM proteins (Samarel, 2005; Okada et al., 2013). Integrin binding recruits a complex of proteins known as a focal adhesion which connect the

cytoskeleton to the ECM and signal downstream to promote a range of cell survival, spreading, and differentiation (Huang et al., 2017a).

The cardiac microenvironment undergoes spatiotemporal changes during development and disease stages (Kapelko, 2001; Bowers et al., 2010; Jourdan–LeSaux et al., 2010; Lockhart et al., 2011; Bayomy et al., 2012; Jung et al., 2012; Hanson et al., 2013). During mouse heart development the ECM is an important component of the cardiovascular progenitor niche (Schenke–Layland et al., 2011). Thimm and colleagues showed elastin enriched regions overlapped with regions with mouse cardiomyocyte differentiation, and observed the inverse correlation with presence of collagen I (Thimm et al., 2015). During fetal–to–adult development, the ECM remodels, shifting from a composition rich in fibronectin to a composition richer in collagen I and laminin (Williams et al., 2014; Rienks et al., 2014; Hanson et al., 2013). Adult ECM contributes to signaling that maintains tissue homeostasis (Williams et al., 2014). However, the composition of the cardiac ECM shifts again during aging or disease with an accumulation in collagen I and decrease in the ratio of collagen III to collagen I (Weber et al., 1988; Kapelko, 2001; Atmanli et al., 2017). Hence, the myocardium stiffness also evolves from softer (few kPa) to stiffer (10 kPa) during development. (Engler et al., 2008, 2007). With age and the onset of cardiac diseases, including dilated and hypertrophic cardiomyopathies, the ECM fraction increases and crosslinks become more pronounced, which are characteristics of tissue fibrosis. Fibrosis changes the mechanical properties of the myocardium, which increased from around 10 kPa to >35 kPa (Bayomy et al., 2012; Wenket et al., 2011; Ho et al., 2010; Conrad et al., 1995; Dean et al., 2005; Berry et al., 2006).

2.4. Soluble signaling factors contribute to remodeling and feedback

In addition to specific adhesion ligands, soluble signaling factors are an important biochemical component of the microenvironment (Euler, 2015; Bergmann, 2010). Many signaling factors (e.g. transforming growth factor, fibroblast growth factor, Wnt, interleukins) are released by cardiac cells and mediate autocrine and paracrine signaling (Melendez et al., 2010; Sakurai et al., 2013; Kong et al., 2014; Ozhan and Weidinger, 2015). Other factors, including both natural compounds (e.g. Angiotensin) and exogenous drugs (e.g. Verapamil) are carried into the heart by the vascular network (Tsutsui et al., 2007). There is significant crosstalk between mechanosensitive signaling through integrins and signaling from soluble factors that helps coordinate development and exacerbate disease (Ross, 2004; Banerjee et al., 2006; Schroer et al., 2014).

Periods of dynamic cardiac remodeling, including both development and disease are generally characterized by increases in soluble signals and dynamically changing mechanical properties. These changes can contribute to a positive feedback loop of adverse remodeling. Ischemia, pressure overload, aging and viral infections are key initiators of cardiac remodeling process (Rienks et al., 2014; Heymans, 2006). Remodeling leads to changes in size, mass, composition and stiffness of the left ventricle, which can in turn activate more mechanosensitive signaling and tissue remodeling (Kehat and Molkenin, 2010; Rienks et al., 2014). These complex and interacting inputs and responses are important features of cardiac disease *in vivo*, but make it difficult to decipher subtle effects

within cells. The development of engineered environments can allow for precise control of different factors of this native environment and improve functional assessment of iPSC-CMs *in vitro*.

3. Microphysiological systems (MPS)

The term MPS encompasses microengineered devices at various hierarchical length scales, ranging from single cells microniches to complex engineered tissues with multiple cell types. Each type of MPS brings its own set of advantages and disadvantages, which we review below (Table 1). The engineering of MPS has provided new means to create *in vitro* model systems that better recapitulate specific properties of the native *in vivo* microenvironment of the heart (Kim et al., 2009; Benam et al., 2015). The higher level of control over cellular and microtissue spatial structures, mechanical properties, and biochemical cues has enabled enhancing the maturity of hiPSC-CMs, as well as reproducing more physiological biophysical behavior (Pasqualini et al., 2015; Sheehy et al., 2017).

Cardiac MPS have been developed and used for more than two decades (Eschenhagen et al., 1997) with other cell systems than iPSC-CMs, in other contexts and under other names, including organ-on-a-chip, heart-on-a-chip, cardiac microtissues, engineered cardiac tissues, engineered human myocardium or even bioMEMS (Huh et al., 2013; Stancescu et al., 2015; Zhang and Radisic, 2017; Conant et al., 2017). There is also a large overlap between MPS designed as *in vitro* cardiac models and other embodiments, such as engineered tissue patches, aimed towards regenerative cardiac medicine. The description of these systems and applications falls beyond the scope of this review and we refer the readers to the corresponding literature (Bouten et al., 2011; Zorlutuna et al., 2013; Capulli et al., 2016; Ogle et al., 2016; Song et al., 2018).

Similarly to Song et al., we distinguish MPS based on the type of approach used to control the cellular organization as: bottom-up, i.e., where the cellular organization is acquired intrinsically through maturation and self-organization of the cellular construct; or top-down, where organization is provided through engineering methods, such as scaffold, structures or patterns (Song et al., 2018). A combination of these approaches is sometimes also found.

In both cases, the engineering effort is set towards improving the physiological function, mostly through the enhancement of the structural and functional maturity of the cardiomyocytes by designing the mechanical properties and biochemical functions of the scaffolds, ligands, and multicellular environment.

3.1. Top-down MPS

Engineering cellular environments with specific spatial, mechanical, and biochemical cues can allow for detailed study of the mechanobiological effects of environment on cardiomyocyte function. However, many of these approaches rely on specialized fabrication equipment and techniques which might not be easily adaptable to every group.

Single cell MPS—In top-down approaches, where the organization is provided through engineering methods, we first consider MPS systems focused on single cells. Small islands

of ECM proteins are patterned using engineering methods including microcontact printing or lithography techniques (Moeller et al., 2018; Ribeiro et al., 2015; Alom et al., 2007). These systems represent an ideal link between *in silico* and *in vitro* molecular model and the more complex multi-cell MPS and engineered tissues (Aratyn-Schaus et al., 2016; Lind et al., 2017b). Patterning ECM serves dual purposes of increasing uniformity within an initially heterogeneous population and, in the case of rectangular patterns, promoting increased alignment of intracellular structures (Ribeiro et al., 2015). Combining ECM patterning on materials with tunable stiffness such as polyacrylamide (Ribeiro et al., 2015; Chopra et al., 2018), PEG hydrogels (Lee et al., 2017), or micropillars (Rodriguez et al., 2014; Tan et al., 2003) provides physiologically relevant mechanical cues and allows for measurement of force generation by single cells. However, there are some notable limitation of these single cell systems, especially when used for relatively immature hiPSC-CMs. These cells lack cell-cell contacts that promote appropriate patterning of sodium channels and gap junctions (Geisler et al., 2010). Without these junctions, it is difficult to study the conduction of calcium currents and action potentials, which are both important features of cardiac electrophysiology. Some microfluidic/microelectrode systems have been used to measure the electrophysiology of isolated primary cardiomyocytes, but the differences in ion channel expression and cytoskeletal organization between primary cardiomyocytes and hiPSC-CMs should temper expectations for acquiring accurate electrophysiologic mechanisms with single cell systems (Werdich et al., 2004; Cheng et al., 2006).

Multiple cells in 2D/3D MPS—Similarly to single cell MPS, some 2D multicellular constructs use ligand micropatterning or surface microstructuring to form planar MPS (Feinberg et al., 2012; Wang et al., 2014; Ariyasinghe et al., 2017). Some MPS, including the muscular thin film, use a combination of micropatterning and lithography based microfabrication to create a series of flexible 2D microtissues whose mechanics are well characterized (Grosberg et al., 2012; McCain et al., 2013; Shim et al., 2012). Other microfabrication techniques including electro spinning have been used for the creation of scaffolds to support engineered tissues (Huang et al., 2017a; Liu et al., 2017; Schenke-Layland et al., 2011; Capulli et al., 2016; Wanjare et al., 2017). These systems allow for top-down engineering of the tissue morphology and extra-/intracellular organization, while simultaneously enabling the study of cell-cell interactions in a microtissue format. Many 2D systems are designed to allow for microscopy and electrophysiologic measurements of coordinated cardiomyocytes. However, cardiomyocytes in these constructs lack the 3D cell-cell interactions which they experience in the native myocardium, which may also limit the ability of these systems to model complex processes like action potential conduction (Lemoine et al., 2017). Methods for top-down engineering of 3D MPS have also been proposed, mostly hedging on 3D printing of scaffolds onto which chemical cues can be spatially patterned (DeForest et al., 2009; Greiner et al., 2012; Scheiwe et al., 2015).

3.2. Bottom-up MPS

Bottom-up MPS are commonly produced by casting of hiPSC-CMs with or without other cells such as fibroblast in a microfabricated mold. The microtissue is typically formed through the combined aggregation of a random hydrogel scaffold and cell-cell contact formation (Mannhardt et al., 2017; Tiburcy et al., 2017; Sidorov et al., 2017; Ronaldson-

Bouchard et al., 2018; Boudou et al., 2012). In most cases, fibroblasts or other mesenchymal cells are added to the hiPSC–CM to form a functional tissue, eventually forming a co–culture. Different geometries have been used for these kinds of constructs, including rings (Tiburcy et al., 2017), sheets (Huebsch et al., 2015), and linear or dogbone shapes mounted on each end (Mannhardt et al., 2017; Sidorov et al., 2017; Ronaldson–Bouchard et al., 2018; Boudou et al., 2012; Leonard et al., 2018). While some have rigid boundaries, many include a flexible component with defined mechanical properties which allows for construct shortening and calculation of force. Many of these systems also include electrodes that allow for direct electrical stimulation of the cardiac construct (Ronaldson–Bouchard et al., 2018; Boudou et al., 2012; Sidorov et al., 2017). While such engineered myocardium tissue better replicate the complex cell–cell interactions found *in vivo*, deconvolving differences in heterogeneous cell populations is more challenging with a large number of cells. Some recent efforts have been made to miniaturize these constructs, reducing the number of required cells per construct from millions to thousands or hundreds (Boudou et al., 2012; Mills et al., 2017). These modifications may make these constructs more scalable for high throughput screening applications.

3.3. Hybrid MPS

Finally, hybrid MPS platform have been proposed that largely overlap with engineered cardiac tissue (Lind et al., 2017a). Most are based on 3D bioprinting, which offers the capability to precisely position single cell and/or scaffold components to form hierarchical tissues including vasculature. The description of such systems fall beyond the scope of our review and we refer reader to the recent literature (Fleischer et al., 2017; Borovjagin et al., 2017). Another strategy relies on laser cutting thin strips of decellularized cardiac tissue and adding hiPSC–CMs to an ECM scaffold with physiologic organization, which in turn promotes cell alignment and maturity (Schwan et al., 2016).

3.4. Designing MPS: choosing materials and ligands

For most MPS, achieving a physiologically–representative extracellular environment is critical, as the mechanical properties and spatial organization of biochemical cues directly impacts the hiPSC–CMs phenotype (Vining and Mooney, 2017). Polydimethylsiloxane (PDMS) is a flexible, bioinert polymer that can be formulated to span a large range of roughly physiological stiffnesses (5 kPa–3 MPa), and it can be easily molded into micro patterned structures (Palchesko et al., 2012). hiPSC–CMs cultured on PDMS grooves or cyclically stretched PDMS sheets demonstrate higher maturity markers and functional characteristics than 2D counterparts (Kroll et al., 2017; Jung et al., 2016). This material is also often used for micropillars, stamps for micropatterning, and as structural material for larger scale MPS, including the deformable anchoring posts of several bottom–up MPS (Boudou et al., 2012; Ronaldson–Bouchard et al., 2018; Ribeiro et al., 2015; Leonard et al., 2018; Rodriguez et al., 2014). Hydrogels are tunable in the range of physiologic cardiac tissue that includes stiffnesses matching early developmental time points and are cell substrate and tissue scaffolds. Various synthetic hydrogel systems have been proposed, based on polyacrylamide (Ribeiro et al., 2015; Chopra et al., 2018), polyethylene glycol (PEG) (Lee et al., 2017), hyaluronic acid (Kloxin et al., 2010), crosslinkable proteins, such as or biosynthesized materials, such as elastin protein (Chung et al., 2012).

While the mechanical properties are important, they alone fail to provide precise control over the tissue and intracellular organization and to spatially mimic the native extracellular matrix. Some MPS focus on coating different ECM proteins on substrate to observe relationship between cell–ECM interaction (Williams et al., 2014; Kong et al., 2013), Williams and colleagues coated a combination of proteins on tissue culture plastic and compared fetal, neonatal, and adult ECM composition. Neonatal ECM lead to greater expansion of neonatal rat ventricular CMs (Williams et al., 2014). Vitronectin–coated–synthetic fibers electrospun on glass coverslips have also shown different synthetic combinations leading to changes in contractility and mitochondrial function in iPSC–CMs (Chun et al., 2015). CMs have been shown to have different spread area, adherence properties and onset of beating depending composition of the ECM proteins (Simpson et al., 1994; Vanwinkle et al., 1996; Bick et al., 1998; Bullard et al., 2005; Deitch et al., 2012). Collagen IV and laminin have been shown to increase hiPSC–CM spread area and contraction velocity on micropillars compared to fibronectin, though there was no statistical difference in force generation between these three (Rodriguez et al., 2014).

Overall, MPS platforms are useful tools for modeling specific aspects of cardiac biology at a range of scales and levels of complexity. For applications where animal models or traditional cell culture approaches fail to replicate relevant features of human cardiac biology, MPS may be a viable alternative. The choice of MPS will be driven by the end application for *in vitro* disease modeling and drug testing.

4. Use of MPS for studying CM biology

A key advantage of many of these MPS over animal models is the ability to precisely tune the mechanical and biochemical environment experienced by the cells and observe the dynamic reorganization of structures and corresponding changing in function and gene transcription.

4.1. Changes in intracellular myofibril organization

Many of these systems are designed to be optically transparent and thus suitable for optical imaging of cells and their internal structures *in situ* (Ribeiro et al., 2015; McCain et al., 2013). Single cell micropatterning studies have discovered that myofibril organization is affected by pattern shape, and that these structures dynamically remodel to align in directions of principal stress associated with clustering of adhesion structures (Parker et al., 2008; Grosberg et al., 2011; Yuan et al., 2017). Alignment in a rectangular shape with an aspect ratio of 7:1 promotes alignment of myofibrils and increased force generation (Ribeiro et al., 2015). Increased resolution, alignment, and registration of myofibrils is a key feature of hiPSC maturation (Leonard et al., 2018). A recent study using tagged alpha–actinin has enabled visualization of centripetal motion of contractile proteins during the re–formation of sarcomeres and myofibrils after replating on patterned substrates (Chopra et al., 2018). In addition to patterning, other features of the engineered substrate can also direct intracellular organization. The available binding proteins and stiffness of the substrate both affect myofibril alignment and resultant force generation (Chopra et al., 2011). Many 3D, bottom up MPSs have been shown to promote better myofibril maturity and density, but

visualization of these structures in live cells is more challenging (Huebsch et al., 2016; Sidorov et al., 2017).

4.2. Effects of cell-cell interactions

Another key feature of the cells microenvironment often mimicked in MPS is the populations of neighboring cells. Pairs of cardiomyocytes have been shown to form adhesions to each other and align their myofibril structure and are capable of generating more force than a single cell alone, according to traction force measurements (McCain et al., 2012). Larger multicellular constructs often include (and indeed require) a population of fibroblasts or other mesenchymal cells for remodeling and maintaining the ECM (Tiburcy et al., 2017; Liao et al., 2017). The ratio and specific types of stromal cell populations added to these constructs can have profound effects on construct properties and CM function. Some studies have relied on the fraction of non-CM cells present in most iPSC-CM cultures, though others have generated a purified culture of CMs and added mesenchymal stem cells or fibroblasts directly at the point of tissue assembly (Hudson et al., 2011; Tiburcy et al., 2017). Tiburcy et al. reported similar peak force production at a final cell ratio of 1:1 non-CM:CM with both mixed iPSC derived cells and purified iPSC-CMs with added fibroblasts (Tiburcy et al., 2017). These mesenchymal cells affect CM function directly (through cell-cell contacts) and indirectly by modulating the ECM stiffness and releasing paracrine signaling factors (Vunjak-Novakovic, 2017; Ariyasinghe et al., 2017; Sidorov et al., 2017). Fetal mouse cardiac mesenchymal cells express more ECM genes and promote a faster beating rate than mesenchymal stem cell derived mesenchymal cells when cocultured with embryonic cardiomyocytes (Hudson et al., 2011). Fetal mouse cardiac fibroblasts promote a more mature phenotype in ESC-CMs than adult cardiac fibroblasts, in part by activating the ERK/MAPK pathway through paracrine signaling (Liao et al., 2017). Some systems have also included endothelial cells, a third major cellular fraction in native hearts. These cells are crucial for providing oxygenation and nutrients for native tissue while also providing important paracrine signaling (Chen et al., 2005; Wanjare et al., 2017). While most MPS are designed to avoid the need for vascularization, applications trending towards therapeutic tissue engineering or more complex organ-on-chip systems will seek to include a vascular bed (Ogle et al., 2016).

4.3. Modeling disease causing mutations

The combination of MPS and hiPSC-CMs allow for the study of effects of mutations which are known or suspected to lead to human cardiac disease in a model of human cardiac cells. This technique has tremendous potential to clarify the underlying etiology of genetic diseases, and to guide decision making about therapies for patients with a specific genetic makeup (precision medicine) (Eschenhagen et al., 2015; Sayed et al., 2016). Many mutations in sarcomeric or structural proteins have been linked to the development of cardiomyopathies, the two most common types of which are hypertrophic and dilated (Fatkin et al., 2014; Spudich, 2014). By promoting better alignment of myofibrils and maturation of contractile machinery, MPS can enable functional measurements of iPSC-CM whose structures are more similar to native CMs. Mutations in sarcomeric proteins, including cardiac troponin C, beta myosin heavy chain and myosin binding protein C change force generation by single hiPSC-CMs, and these data in conjunction with data from *in vivo*

experiments support contractile tension as a predictive indicator of the development of dilated or hypertrophic cardiomyopathy (Davis et al., 2016). Separately, traction force measurements on a micropatterned platform showed reduced force production and increase spatial heterogeneity of contraction from single hiPSC–CMs with myosin binding protein C mutations (heterozygous and homozygous deletion) in a dose dependent way (Ribeiro et al., 2017). Single cell analysis of cells with mutation of BAG3, a chaperone protein associated with the development of DCM, also revealed reduced contractility (Judge et al., 2017). Muscular thin films have been used to describe functional differences in hiPSC–CM contraction and myofibril organization with a mutation for Barth Syndrome, a mitochondrial condition (Wang et al., 2014). A titin mutation reduced force generation and normal myofibril formation when compared to WT differences in both micropatterned single cell platforms and 3D engineered heart tissue constructs (Chopra et al., 2014; Hinson et al., 2015; Chopra et al., 2018). These studies can clarify the mechanisms of disease progression from specific genetic causes and may eventually inform treatment choices for specific patients.

5. Drug discovery

The use of hiPSC–CMs as models to develop drugs for therapy and to screen compounds for cardiotoxicity have been proposed since their advent over a decade ago, but progress has been limited by the relative immaturity of these cells (Zhang et al., 2009). Since MPS enhance the maturity of hiPSC–CM, their utilization for drug discovery has increased (Huebsch et al., 2016; Ewart et al., 2018). Increasing maturity of hiPSC–CMs is correlated with the expression of specific beta adrenergic receptors, which are necessary to reproduce physiologic responses to norepinephrine (Tiburcy et al., 2017). Culturing cells on micropatterned PDMS substrates accelerated maturation by more than 6 weeks and promoted expression of beta adrenergic receptor 1 that enabled the an adrenergic response (Jung et al., 2016). However, there are still limitations to using MPS such as engineered heart tissue (EHTs) or single cell systems for drug discovery. Throughput and scalability are critical for drug discovery, but most EHTs require a relatively high amount of cell (>1 million cells/EHT) for one EHT and at least a week to develop (Eder et al., 2016). There have been efforts to miniaturize these constructs, which can improve the scalability for drug discovery applications and multi-factorial analysis (Mills et al., 2017; Boudou et al., 2012). In contrast, single cell systems can provide high-throughput, but have yet to demonstrate the maturity seen in EHTs (Ribeiro et al., 2015). Despite this lack of maturity, micropatterned single hiPSC–CMs can recapitulate the effects of cardioactive compounds isoproterenol and caffeine on CM structure and function (Ribeiro et al., 2017). To date, most studies that have used hiPSC–CMs to screen for cardiotoxicity or drug development have examined the impact of these compounds on the electrophysiological properties of the cell (Sayed et al., 2016; Burridge et al., 2016; Sala et al., 2016). These examinations are fully warranted, as disruption in the electrical properties of CMs by several trial drugs have led to long QT syndrome (Gintant et al., 2017). However, examination of the impact of drugs on force production and contractility is also necessary, as drugs that are deleterious to either of these parameters may reduce cardiac function. Thus, the future of MPS for drug discovery will need platforms that can provide electrical and functional data in a high throughput platform.

6. Conclusión

Overall, this field has expanded with the growing accessibility of hiPSCs and low-cost differentiation strategies. However, the question remains open to what degree the immaturity of iPSC-CMs in traditional culture systems limits their utility for disease modeling or drug discovery. There are a range of MPS that are currently in use for studying hiPSC-CM maturation and recapitulation of both healthy and diseased myocardial tissue function.

Larger cardiac tissue constructs are also being investigated as development of directly therapeutic cardiac patches for personalized tissue engineering, though these currently focus on generic (not patient specific) stem cell derived CM cell sources (Chong et al., 2014). While questions of regulatory approval and fast, reliable iPSC cell creation and differentiation may slow the adoption of iPSC-CM use in direct therapeutic applications, a better understanding of iPSC-CM function and microenvironment may have many important implications for developing future therapies for heart disease.

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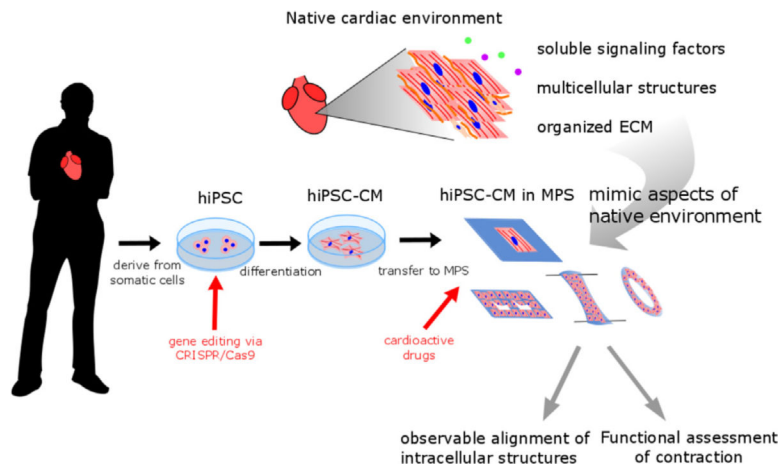


Fig. 1. Microphysiological systems (MPS) mimic aspects of native cardiac environment to enable disease modeling and drug discovery using human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs).

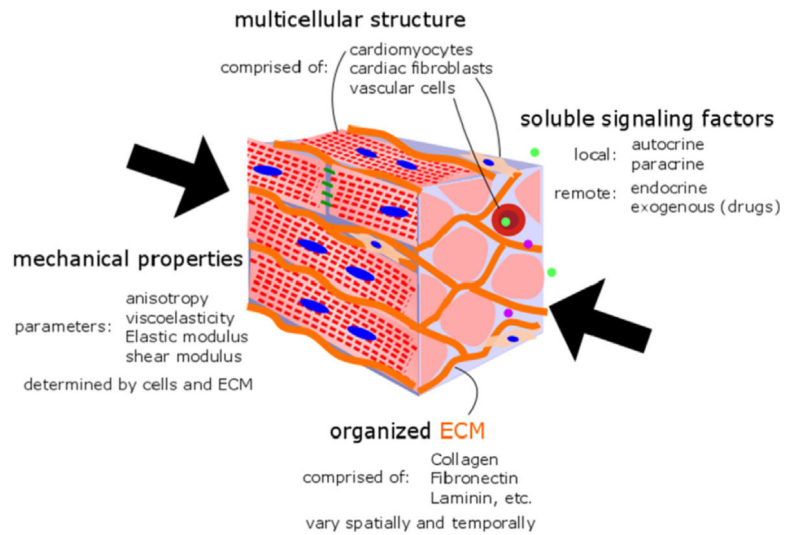


Fig. 2. Native myocardial microenvironment can be characterized by mechanical properties, cell populations, extracellular matrix components, and soluble signaling factors.

Table 1

Summary and comparison of MPS systems used for functional assessment of hiPSC-CMs. For each type of MPS systems, the controllable features and measurable output are described as reported (+) or not reported (-). The number of replicates possible to derive from 10⁶ cells is a theoretical maximum based on the loading conditions described in the representative dted studies, though for many of the smaller scale constructs, the number of replicates is more limited by the later data-acquisition processes.

Approach Type	MPS	Controllable features			Measurable outputs			Scalable for high throughput studies	Ref.
		Spatial structures	Mechanical properties	Biochemical cues	Mechanical function	Electrophysiology	Visual observation		
		Cell	Tissue					number of replicates that can be made from 10 ⁶ cells	
Top-Down	Single cell	+	-	+	+	+	+	+	(McCain et al., 2012; Ribeiro et al., 2015; Chopra et al., 2018)
	Micropatterned substrates								$(5 \times 10^3 - 5 \times 10^5)$
2D Multicell	Microfluidic & microelectrodes	possible	-	possible	possible	+	+	+	Werdich et al., 2004; Cheng et al., 2006; Qian et al., 2017)
	Micropatterned and microstructured substrates	+	+	+	+	+	+	+	$(5 \times 10^3 - 5 \times 10^5)$
Bottom up	Microfluidic heart-on-chip	possible	possible	+	+	+	+	+	(Stancescu et al., 2015; Alford et al., 2010; Grossberg et al., 2011; Shim et al., 2012; Feinberg et al., 2012; Lind et al., 2017b; Kujala et al., 2016)
	EHTs	-	+	-	+	+	-	+	$(2-20)$
Hybrid	3D bioprinting/recellularized	-	+	+	+	+	-	+	(Marsano et al., 2016; Mathur et al., 2015, 2016)
									$(0.5-200)$

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Approach Type	MPS	Controllable features	Measurable outputs	Scalable for high throughput studies	Ref.
		<p><u>Spatial structures</u></p> <p>Cell Tissue</p> <p><u>Mechanical properties</u></p> <p><u>Biochemical cues</u></p> <p><u>Mechanical function</u></p> <p><u>Electrophysiology</u></p> <p><u>Visual observation</u></p> <p>In situ End-point analysis</p>	<p>number of replicates that can be made from 10⁶ cells</p>		
		cardiac ECM scaffolds			