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Electrical and mechanical stimulation of cardiac cells and tissue constructs☆

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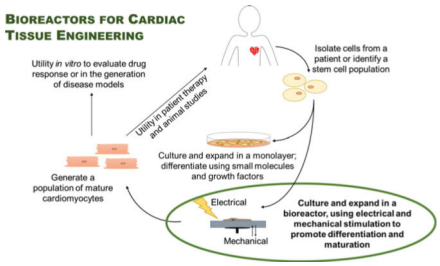
Abstract

The field of cardiac tissue engineering has made significant strides over the last few decades, highlighted by the development of human cell derived constructs that have shown increasing functional maturity over time, particularly using bioreactor systems to stimulate the constructs. However, the functionality of these tissues is still unable to match that of native cardiac tissue and many of the stem-cell derived cardiomyocytes display an immature, fetal like phenotype. In this review, we seek to elucidate the biological underpinnings of both mechanical and electrical signaling, as identified via studies related to cardiac development and those related to an evaluation of cardiac disease progression. Next, we review the different types of bioreactors developed to individually deliver electrical and mechanical stimulation to cardiomyocytes *in vitro* in both two and three-dimensional tissue platforms. Reactors and culture conditions that promote functional cardiomyogenesis *in vitro* are also highlighted. We then cover the more recent work in the development of bioreactors that combine electrical and mechanical stimulation in order to mimic the complex signaling environment present *in vivo*. We conclude by offering our impressions on the important next steps for physiologically relevant mechanical and electrical stimulation of cardiac cells and engineered tissue *in vitro*.

Graphical Abstract



☆This review is part of the Advanced Drug Delivery Reviews theme issue on "Tissue engineering of the heart: from in vitro models to regenerative solutions".

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1. Introduction

Cardiovascular disease is the leading global cause of death, accounting for 17.3 million deaths per year, and is responsible for about one in every three deaths in the United States [1]. Following significant injury such as myocardial infarction (MI), the myocardium has limited regenerative potential and, over time, a dense collagenous scar replaces the contractile tissue, which not only cannot contribute to the pumping function of the heart, but also impedes long-term function. To compensate, the remaining viable myocardium hypertrophies, the infarct wall thins, scar tissue replaces the viable myocardium, and the left ventricle (LV) dilates, all ultimately leading to heart failure (HF). As medical management tools such as stents and pharmaceuticals advance, almost 85% of patients survive their first heart attack, yet the 5-year survival rate is only 50% [1], suggesting that further improvements in methods to manage and treat subsequent HF are necessary. Current state-of-the-art treatments for end-stage HF primarily include the application of LV assist devices (chronic use of an external device which was designed as a bridge to transplant [2–4]) and total heart transplantation (limited by donor organ shortage [1,5]), which both require invasive surgeries and some form of immunomodulation. Therefore, there is a pressing clinical need for new therapies to ameliorate the deleterious responses post-MI that lead to HF, including negative left ventricular (LV) remodeling, arrhythmias, and diastolic dysfunction [6].

Cell-based therapies for cardiac repair have taken two forms: 1) Cardiomyoplasty and 2) the generation of a functional cardiac graft for implantation on or in the heart. Cardiomyoplasty has demonstrated substantial promise *in vitro*, as well as in repair in animal models [7–16], but clinical results thus far have shown limited success, often due to poor cell survival and retention [14,17,18]. In addition, when using cell types such as skeletal myoblasts, poor electrical integration into the host tissue can lead to secondary complications such as arrhythmias [19]. The use of tissue-engineered grafts has also shown significant promise in small animal models [20–24], but concerns about cell/ graft viability and the level of cell maturity have limited the clinical potential of tissue-engineered grafts. Future success and development of cardiomyoplasty- and cardiac graft-based treatment strategies would benefit from optimized *in vitro* methods to evaluate cardiomyocyte (CM) viability, phenotype, maturation level, and contractility under varying conditions that mimic the *in vivo* cellular environment. In particular, significant effort should be made to understand the use of induced pluripotent stem (iPS) cell-derived CMs *in vitro*, focusing on methods that aid in maturation, as differentiated iPS cells are functionally closer to a fetal CMs as opposed to an adult CMs. This is particularly true given the ever-increasing push to utilize iPS cell-derived CMs as a cell source in tissue engineering and regenerative medicine applications, as well as in the development of so-called organs or bodies on-a-chip systems. Moreover, the development of systems to mimic the biophysical environment present *in vivo* could be of

significant use in drug discovery and development studies where CMs that mimic the functional phenotype present in adult tissue would be a valuable asset.

To achieve greater CM maturation and function *in vitro*, many researchers have sought to understand the role of mechanical and electrical stimulation in CM gene and protein expression. There has been a significant amount of effort in development of culture platforms that improve CM function compared to traditional 2D culture, where CMs do not align and remain relatively immature [25–27]. The addition of mechanical stimulation can increase maturation and contraction via hypertrophic pathways and the addition of electrical signaling leads to enhanced cell-cell coupling and improved calcium handling. Even with these improvements, current tissue engineering strategies still result in constructs that are functionally dissimilar to native myocardium. Engineered tissues are only able to achieve contractile stresses on the order 5 kPa [27–31], while native human heart tissue is on the order of 15-30 kPa [32]. As such, dual stimulation methods have been implemented which aim to combine both mechanical and electrical stimulation in a physiologically relevant way [33–36], but optimization of these platforms is still required.

In this review, we outline the efforts to improve CM function *in vitro* using mechanical and electrical stimulation, paying close attention to system design and the level of control of cell phenotype. First, the motivation for mechanical and electrical stimulation in the context of cardiac tissue development is discussed, followed by the cell culture and bioreactor systems that have been developed to promote functional CM phenotypes, where mechanical, or electrical stimulation are used individually. Herein, 2-dimensional (2D) and 3-dimensional (3D) culture systems are both reviewed, where the definition of a 2D system is one where CMs are grown on top of a substrate as compared to within (3D) the substrate or biomaterial. We close with a review of strategies for combining electrical and mechanical stimulation in physiologically relevant ways before concluding with a discussion of areas that remain to be addressed by those in the field.

2. Biological basis for mechanical stimulation

Beating, or the generation of contractile force, is a key component of both cardiac development and general cardiac function [37–40]. In humans, the heartbeat of a fetus is measured during pregnancy and the rate of the heartbeat is often used as a marker of fetal health and development. In adults, changes in heart rate or beat frequency can indicate disease, especially noticeable during a heart attack or in patients with arrhythmias. In order to better understand the biological foundation for these basic observations, investigators have utilized animal models, demonstrating that the course murine heart development is similar to that of the human, thus enabling the utilization of mice in the study of genetic and developmental abnormalities, specifically those related to changes in mechanical forces and cardiac specific gene expression [39–42]. Research has shown that bulk mechanical properties of the ventricular tissue change as the animal ages, suggesting that local structural changes, such as ECM crosslinking density, tissue composition, and cell-extracellular matrix (ECM) interactions play key roles during development [42–44] and changes in the aging heart or in disease models suggest that structural changes post-development are signs of cardiovascular disease [45].

These differences observed at the tissue level are also detectable at the cellular level. Changes in basic cellular processes, such as gene expression, protein expression, and cellular communication are influenced by changes in intracellular tension and/or extracellular stress. Specifically, changes in stiffness are transmitted via integrin binding, receptor tyrosine kinase activation, and GTPase activation at the cell membrane, in turn affecting signaling pathways involving important proteins such as Rho/ROCK (Rho-associated protein kinase) (95–97), MAPK (mitogen activated protein kinase)/ERK (extracellular signal-regulated kinases) [46–49], and Akt [50–53]. Rho/ROCK activation can mediate hypertrophy [54–56], glucose and fatty acid metabolism [46, 57–61], oxidative stress [62], focal adhesion kinase (FAK) activation [63,64], proliferation [37,65–67], apoptosis [59,68–70], differentiation [71], and maturation [72,73] in CMs (see [74–76] for reviews and perspective on clinical implications). In addition, cardiac fibroblasts (CFs), are also affected by mechanical stimulation, demonstrating changes in migration [77], ECM expression [78], differentiation, and myofibroblast activation [79–83] following Rho/ROCK activation. MAPK and ERK activation can mediate hypertrophy [84–92], calcium handling [93], oxidative stress [94–97], remodeling [98], proliferation [99], apoptosis [100–105], and maturation in CMs [106–108]. CF response to oxidative stress [109] is also mediated by MAPK and ERK activation. In addition, integrin activation by mechanical stretch can also lead to phenotypical changes in cells through activation and expression of receptor tyrosine kinases. The duration of the stretch-induced signal affects signal propagation via growth factor activation of tyrosine kinases [66,110–116]. For example, integrin binding affects proliferation via the expression of cyclins [115,117], which are key proteins needed for progression past the G1 phase of the cell cycle. Broadly, mechanical stimulation and changes in cytoskeletal tension can affect proliferation, apoptosis, metabolic rates, and gene expression and, more importantly, mis-regulation of these pathways can lead to birth defects and disease.

2.1. Mechanical forces in cardiac development

Mechanical forces also play key roles in development and cardiac tissue morphogenesis. The heart is the first major organ system to form, beginning with the formation of the double-walled primary heart tube from the cardiogenic mesoderm. In the mouse, the linear heart tube begins beating about three weeks following conception (~6 weeks for the human), long before the structure is fully defined and organized into the four-chambered heart. In order to create the four-chambered heart, the linear heart tube undergoes a complex looping process, which follows a strict schedule over the course of human development, continuously changing the topography and physically altering the cellular architecture [118–120]. Nodal- and actomyosin-mediated changes in cell shape are partially responsible for this bending and looping as demonstrated by inhibition of actin polymerization [120,121]. The bending during loop formation or ballooning during chamber development cause cells on the outer surfaces to form elongated convex architectures while cells on the inner surfaces remain cuboidal in shape [122,123], suggesting that changes in cell fate decisions are partially driven by changes in cell shape given that these cell shape changes happens prior to differentiation and maturation. CM contraction and blood flow exist within the primitive heart tube prior to complete chamber development and investigations using Zebrafish have demonstrated that these forces (beating and flow) or alterations in these

forces influence chamber development via changes in cell elongation [123]. Further efforts to genetically or pharmacologically inhibit these processes [37,120,122–124] suggest that changes in flow, shear stress, and cell shape all greatly influence the cardiac developmental process. From an engineering standpoint, it is important to recognize how 2D and 3D structures provide some or all of these cues to cells, especially when differentiation, maturation, alignment, and organization are critical downstream considerations for construct design.

2.1.1. The role of mechanical forces in the development and progression of congenital heart defects—Improper mechanical signaling from surrounding tissue can lead to the development of congenital defects, which may result in lethality prior to birth or the need for postnatal reconstructive surgery. For example, altered blood flow through the developing heart or excessive pressure from translocations of the abdominal organs into the thoracic cavity due to congenital diaphragmatic hernia (CDH), can lead to lethal or severely disabling congenital heart defects such as hypoplastic left heart syndrome (HLHS) [125–127]. *In vivo* studies of these processes have been challenging, given that it is difficult to create animal models that closely mimic human CHD pathologies, many genetic knockouts lead to premature lethality, and often the phenotypic response in the rodent varies greatly from human pathology [128–130]. The ligation of the left atrium in the developing chick embryo is the most widely used model for left heart defects, broadly demonstrating that decreased CM proliferation is a critical factor in disease progression [131]. Nitrofen-induced CDH in rats leads to a high proportion of fetuses that have CHDs similar to that observed in humans [132]. As with the chick model, CM proliferation is decreased in CDH-related CHDs in rats [133]. CMs also have decreased expression of the transcription factors GATA4 and GATA6 [134], which are important in CM proliferation and maturation [135,136]. Furthermore, procollagen and tropoelastin gene expression are reduced [137], suggesting that altered ECM synthesis occurs in the progression of left ventricular disease. However, a change in mechanics resulting from altered ECM composition or expression has yet to be fully evaluated and remains an area of current research interest.

2.2. Mechanical forces in normal and pathological hypertrophic cardiac growth

One of the main outcomes from mechanical stimulation of cardiac cells or tissues is cardiac hypertrophy, which is an increase in CM size within the heart (as opposed to hyperplasty, which is an increase in CM number in the heart via proliferation). Physiological hypertrophy, or an increase in cell mass within the heart during normal processes that include continual exercise (e.g., athlete's heart), normal post-natal development, or pregnancy, is characterized by normal cardiac morphology, with an enhancement in overall muscle function [138]. Most importantly, with a decrease in exercise or the birth of a baby, physiological hypertrophy is reversible. Comparatively, pathological hypertrophy is abnormal cardiac growth in response to disease (e.g., hypertension) that is non-reversible and is characterized by increased interstitial fibrosis, increased apoptosis, and overall cardiac dysfunction [138]. Pathological hypertrophy is a key risk factor for heart failure, and therefore current research aims to understand ways to reduce, reverse, or limit pathological hypertrophy as well as attempts to elucidate the mechanisms behind the pathways that are responsible for the change in phenotype. Pathological and physiological hypertrophies have

different functional, structural, metabolic, and molecular mechanisms. One current area of research interest is aimed at elucidating the mechanisms responsible for physiological hypertrophy and determining methods to alter hypertrophic pathways with the goal of aiding patients with pathological hypertrophy and heart failure by reversing some of the deleterious effects (see Bernardo et al. for review [138]).

Mechanical forces play critical roles in the activation of the pathways resulting in cardiac hypertrophy, with the activation of Rho/ROCK, Akt, MAPK, ERK, JNK, and stress-activated protein kinase (SAPKs) [54–56,84–92,138–149]. Chronic exercise, such as weight-lifting or wrestling, result in concentric hypertrophy [138,143] while endurance training and pregnancy result in eccentric hypertrophy [142,150,151], both of which are reversible when the stimuli end. In addition, physiological hypertrophy is specifically characterized by the expression of p110α phosphatidylinositol-3 kinase (p100α PI3K) and downstream targets [152–154]. Upregulation of secreted small molecule (e.g., hormones) and growth factor expression has been reported in animal models of physiological hypertrophy (e.g., adrenomedullin and insulin-like growth factor-1 (IGF-1)) [155,156].

Pathological hypertrophy represents a reinstatement of the developmental gene program, leading to hypertrophic growth that follows similar mechanisms to those activated during development [85,157]. Pressure overload caused by hypertension or aortic constriction leads to an increase in heart wall thickness and a reduction in the ventricular cavity volume (concentric hypertrophy) via an increase in the number of sarcomeres in parallel, which increases CM width [138,143,158]. In pathologies resulting in volume overload, such as valve disease, the heart wall thins and the ventricular cavity volume increases (eccentric hypertrophy) via addition of sarcomeres in series, increasing CM length, which poses a greater risk to patients comparatively [159]. Pathological hypertrophy in animal models (e.g., aoritic banding or stenosis) is characterized by increased expression of atrial natriuretic peptide (ANP) [160], B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC) [160] and α-skeletal actin, and decreased expression of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) [160] and α-MHC compared to controls [138], which are not seen in models of physiological hypertrophy [161–163]. An increase in heart weight to body weight and lung weight to body weight ratios are common in animal models of pathological hypertrophy [164,165]. A continual understanding of the differences between pathological and physiological hypertrophy, and in particular the role of mechanical stimuli, could lead to therapies for patients with cardiovascular disease [138,143].

3. Biological basis for electrical stimulation

Coordinated contraction of the heart and maintenance of the heart rhythm is controlled by a complex network of interconnected CMs (Fig. 1D), which communicate via gap junctions between neighboring cells and though voltage gated ion channels that control internal and external ion levels. On average, only 1% of CMs within the heart regularly contribute to controlling electrical conductivity and conduction rate, and these CMs are broadly termed pacemaker cells. Pacemaker cells are responsible for generating electrical impulses or action potentials that maintain the electrical connectivity across the tissue [166–170]. On the tissue level, changes in electrical coupling in the heart lead to arrhythmias, which can be fatal if

left untreated [171–173]. For the development of tissue constructs for regenerative medicine, it is critical to consider electrical integration of the construct with the host tissue [174–177], to prevent secondary complications related to graft and host asynchrony [8,178]. Similarly, development of *in vitro* cardiac tissues for drug testing requires electrical interconnectivity to understand how a given drug will affect heart rate or the propensity to develop arrhythmias. This section will highlight the current understanding of the mechanisms and pathways responsible for creating and maintaining cardiac electrical signaling as determined from both developmental investigations and studies of adult pathologies that lead to heart failure.

3.1. Formation of electrical conduction pathways in cardiac development: the role of cell-cell junctions

The role of electrical signaling in cardiac development is typically studied in model species, such as zebrafish or mice. While the zebrafish heart is not comprised of 4-chambers, electrical conductivity between the two-chambered heart is required for proper development and displays similar conduction mechanisms to humans [179–183]. Electrical impulse propagation is regulated primarily through gap junctions, which synchronize action potentials and play an important role in the development of regular synchronous contractions [184]. Specifically, the family of gap junction protein connexins (Cxs) are important in cardiac development and mis-regulation of Cx expression or posttranslational modifications in the structure can lead to fatal arrhythmias or perinatal lethality during development [185,186], (see Saez et al. for a review on connexins [187]). Likewise, control of or modifications to Cxs may also provide a clinical route for reduction of arrhythmogenic substrates, enhancements in cardiac function, and stimulation of remodeling following injury [185]. Specifically, in a zebrafish model, Cx46 is expressed by pacemaker cells and knockout mutants exhibit asynchronous contraction of the ventricular chamber and disorganization of the chamber wall tissue [180]. In Cx46 knockouts, complete rescue of ventricular conduction and contraction occurred in 56% of the animals with the delivery of exogenous Cx46 mRNA and 100% of the knockouts recovered when myocardial specific expression of a Cx46 transgene was utilized [180]. Therefore, Cx46 is critical for the development of physiological electrical currents through gap junctions in zebrafish that affect CM morphology, ultimately impacting cardiogenesis [180].

Cardiac tissue and CMs from mice express a variety of Cxs, including Cx30, Cx30.2, Cx40, Cx43, Cx45, and Cx46 [180,185,186,188,189]. *In vivo* mouse experimentation demonstrates that both the presence and expression patterns of these gap junctions are critical for proper cardiac development [186,188–194], and mis-regulation can lead to developmental defects prior to birth as demonstrated by murine knockouts as well as the evaluation of human patient data [186,192–200]. For example, Cx45 is one of the first Cxns expressed and a Cx45 knockout in mice was used to determine its role in murine heart development. The hearts in $Cx45^{-}$ animals began to contract on embryonic day nine; however, within a few hours, a conduction block appeared and the myocardial walls displayed an endocardial cushion defect, even though the cardiac jelly was present [186]. Further analysis showed that Cx45 is critical for epithelial-mesenchymal transformation of the cardiac endothelium via signaling through Ca^{2+}/calcineurin and NFATc1. Importantly, this work demonstrates that

gap junction activation and changes in expression patterns are critical for the development of the conduction system in the heart [186,201]. The implication of these findings suggests that differentiation and maturation of CMs, which is characterized by the presence of tight, functional gap junctions, integrin binding, and intracellular tension, require both mechanical and electrical stimulation for proper physiological development, motivating much of the research reviewed below (Sections 4 and 5).

3.2. Electrical signaling in cardiomyocyte differentiation and maturation and the development of pathologies

In mature cardiac tissue, electrical signal activation is regulated by exchange of ions between the intra- and extracellular spaces via voltage-gated ion channels (e.g., sodium channels (Nav1.5) or potassium channels (Kir1.2, Kv4.2)) and conduction of these signals is propagated via gap junctions. Human induced pluripotent stem cell (hiPSC)-derived CMs lacking voltage-gated ion channel expression are unable to mature into functional myocytes [202]. In addition, alterations in expression levels or mutations in voltage-gated ion channels and Cxs have been reported in cardiac disease and are associated with the development of arrhythmias [166,203–207]. For example, investigation of a murine model of calcineurin-induced hypertrophy demonstrated a down-regulation of Cx40 and Nav1.5, leading to the development of arrhythmias and increased mortality [203]. In addition, the analysis of patients with dilated cardiomyopathy presenting with ventricular tachycardia as the initial and cardinal manifestation of the disease were found to have mutations in the KCNQ1 gene, which codes for a voltage-gated potassium channel [207]. These results demonstrate that alterations to the expression of voltage gated ion channels, which impacts ability of CMs to respond to electrical stimulation and activation, play a crucial role in a number of potential disease states. Improper signaling can lead to cardiac disease and undesired consequences, thus demonstrating the critical need to consider cell–cell connectivity and ion channel expression when designing 3D models for drug testing or scaffolds for cardiac repair.

4. Mechanical stimulation of cardiac cells and tissues

The heart starts functioning early in development and, thus, much of the growth and reorganization that occurs in utero is in the presence of changing mechanical stimuli. Moreover, alterations to cardiac function with disease usually result from or cause alterations to the mechanical function of the organ, which alters the stress present on CMs. As a result, there has been a concerted effort to assess the effects of various mechanical stimuli on CMs *in vitro*, in both two-dimensional (2D) and three-dimensional (3D) culture. The primary methods for mechanically altering cells are: 1) passive material stiffness, 2) step-wise stretching to increase strain over time, and 3) dynamic stretching where the cells are stretched and relaxed at a given frequency (Fig. 2). The goal of these manipulations is to maintain and promote phenotypes characteristic of mature CMs (Fig. 1). The above-mentioned methods have also been used to elucidate the role of mechanical signaling in hypertrophic signaling in both a physiological and pathological context [208,209]. In this section, a review of the relevant literature focuses on efforts to enhance CM function and phenotype via mechanical stimulation as well as studies aimed at utilizing mechanical

stimulation as a tool to understand hypertrophic signaling in CMs (Note: for a more complete review, see Zimmermann et al. in this issue or Tallawi et al. [210]).

4.1. Mechanical stimulation in 2D

In studies involving two-dimensional (2D) culture of CMs, mechanical forces have generally been applied through two distinct mechanisms: 1) materials with varying mechanical properties have been utilized to affect intracellular tension (Fig. 2A), and 2) the substrate on which the cells are grown has been mechanically strained either in a step-wise fashion (Fig. 2B) where the material is stretched in incremental steps that are held for extended periods of time, or under cyclic stretch where the construct is stretched and relaxed at regular intervals (Fig. 2C). In all cases, outputs of interest include cellular alignment/sarcomere organization, alterations in gene and protein expression relevant to CM phenotype/function and calcium handling/contractile properties of the CMs.

4.1.1. Substrate mechanics and cardiac phenotype—Mechanical analysis of excised cardiac tissue demonstrates that tissue stiffness changes throughout the course of development [42,211] and changes to tissue properties in diseased tissue impacts contraction force [32]. Atomic force microscopy measurements of developing murine heart tissue showed that the tissue mechanics stiffen drastically after birth (embryonic/fetal: 12 ± 4 kPa, neonatal: 39 ± 7 kPa) [42], which is in agreement with uniaxial tension measurements (embryonic/fetal: ~10 kPa, neonatal/adult ~20 kPa) [211]. Similarly, using micropipette aspiration the elastic modulus of healthy neonatal rat heart tissue was determined to be 4.0 to 11.4 kPa (mean value of 6.8 kPa) and healthy adult rat heart tissue to be 11.9 to 46.2 kPa (mean value of 25.6 kPa) [212]. Given that these changes in tissue stiffness occur simultaneously with alterations in CM growth and function [42,211], it is likely that isolated CMs plated on substrates of varying material properties will alter protein or gene expression and cellular phenotype, enabling the use of stiffness as a tunable property for manipulating CM function *in vitro*.

4.1.1.1. Substrate stiffness and cardiomyocyte maturation and function: To evaluate the role of substrate stiffness on alignment of CM striations independently from ligand binding density, Jacot et al. utilized polyacrylamide gels (1, 10, 50 kPa) coated with 0.5 mg/mL rat-tail collagen I via covalent coupling [213]. Neonatal CMs had well defined and aligned striations on the 10 kPa gels, similar to embryonic tissue, while CMs exhibited unaligned striations with long, large stress fibers on stiff 50 kPa gels, suggesting that the use of substrates *in vitro* that do not match *in vivo* mechanics may lead to a cellular response that is not characteristic of *in vivo* function. Neonatal CMs also had the greatest action potential response on a material stiffness that matched that of the embryonic heart *in vivo* [42]. In another study, collagen I coated PAAm gels were also used to determine the relationship between cellular strain and matrix strain during contractions in embryonic chick CMs [214], demonstrating that soft matrices allowed the CMs to deform the matrix while stiffer substrates only allowed the CMs to deform themselves. In a similar investigation, isolated neonatal rat cardiac cells were seeded onto collagen-coated polyacrylamide gels with Young's moduli of 3, 22, 50, and 144 kPa [212]. After 120 hours, CMs on 3 Pa gels had the lowest excitation threshold (3.5 ± 0.3 V/cm) and increased cTnI staining (52% positively

stained area), but reduced cell density, force of contraction (0.18 ± 0.1 mN/mm^2), and CM elongation (aspect ratio = 1.3–1.4). The 144 kPa collagen-coated PAAm gels exhibited reduced cTnI staining (30% positively stained area), increased CF density (70% positively stained area), and poor electrical excitability. Overall, these results showed that collagen-coated substrates that are stiffer than healthy cardiac tissue (~50 kPa) impede maturation of isolated CMs *in vitro*, while softer substrates that mimic the mechanical environment of heart tissue at earlier stages in development can enhance some aspects of CM maturation.

Collagen I is an abundant protein the extracellular matrix of adult heart tissue, but the basement membrane that CMs bind to contains a significant amount of laminins. As such, a variety of studies assessing mechanotransductive signaling in adult CMs have utilized laminin-coated substrates and report somewhat different findings from the collagen coated substrates described above. Adult CMs seeded on soft, compliant (7 kPa) laminin coated polydimethylsiloxane (PDMS) substrates exhibited organized sarcomeres after 48 hours, but sarcomere organization was lacking on materials with a mid-range stiffness (117 kPa, 27 kPa) [215]. However, adult CMs seeded on very stiff (255 kPa) laminin coated PDMS substrates exhibited organized sarcomeres [215]. Interestingly, evaluation of α-actinin protein levels showed that expression was equal across all stiffnesses [215], suggesting that stiffness did not affect α-actinin protein production, but did influence intracellular protein organization. Gene expression via RT-qPCR showed an increase in α-actinin mRNA expression on 27 kPa gels in addition to upregulation of integrin β1 and vinculin, suggesting that CMs on 27 kPa were trying to recover lost function and organization during the first 48 hours of *in vitro* culture [216]. These results, among others, suggest that changes in substrate stiffness affect gene expression and production of functional proteins, as well as affecting intracellular activity and protein post-processing, such as phosphorylation, ubiquitination, and degradation [217–221].

Studies have also been carried out to assess the effects of 2D substrate stiffness on CM contraction rate, stress generation, and intracellular calcium handling [215,222–225]. Galie et al. showed that not only did stiffness affect the stress generated within the CMs, but also the level of strain. In addition, there was a temporal change in these parameters suggesting that the CM response *in vitro* is an adaptive, time-dependent process [215]. Bajaj et al. showed similar results which demonstrated that within the first 24 hours, the substrate stiffness had a profound effect on the beating frequency of embryonic chick CMs plated on laminin-coated PAAm gels (18 kPa was greater than 1 kPa, 50 kPa, and tissue culture plastic) [226]. However, after five days, the CM response was not dependent upon the stiffness of the substrate, suggesting that CM attachment, function of native CFs within the isolated population, and establishment of cell-cell contacts can overcome the initial shock of 2D *in vitro* culture. Future work should correlate these measurements with the structure and density of focal adhesions, as this temporal change in CM contractility over the first few hours could be partially attributed to the level of attachment to the substrate itself as well as the role of remodeling by native CFs found in neonatal and adult CM isolations. Overall, these results and others demonstrate that stiffness plays a role in CM maturation (Fig. 1C) *in vitro*, which can be affected temporally and via changes to growth factor or small molecule

delivery [215,222–224,226,227]. Stiffnesses around 10 kPa or above 200 kPa lead to the most organized sarcomeric structure after short culture times (≤48 hours).

4.1.1.2. Substrate stiffness and cardiomyocyte differentiation: Stem cell differentiation is affected by both substrate stiffness and ligand density, suggesting that these two external cues impact cellular maturation via changes in gene and protein expression for a variety of cell types [214, 228–230]. Many studies have evaluated the role of soluble factors in the differentiation and maturation of stem cell-derived CMs, demonstrating that stem cell-derived CMs increase in maturation over time in culture [231–234]. The ability of substrate stiffness to aid in the differentiation of stem cells [229,230,235,236], suggests that utilization of substrates with stiffnesses, which match the changes present in development, during the *in vitro* growth, differentiation, and maturation of stem cells into CMs may lead to improved differentiation in shorter culture periods. Indeed, in a study of the response of human stem cell-derived CMs to substrate stiffness, cells were seeded on polyacrylamide gels containing fluorescent beads for analysis with traction force microscopy [222]. Stem cell-derived CMs exhibited statistically higher average contraction stress on 100 kPa gels in comparison to isolated neonatal CMs, while measurements at all other stiffnesses were statistically similar (4-76 kPa), suggesting that 100 kPa gels enhanced the functional maturation of the stem cell-derived CMs.

Evaluation of micropatterned substrates has also shown that the topography or pattern on 2D substrates can influence stem cell-derived CM alignment [237,238]. Stem cell-derived CMs cultured on fibronectin-coated polydimethylsiloxane microgrooved substrates (10 μm apart, 10 μm wide, 4 μm deep) for two weeks, demonstrated improved cellular alignment ($p < 0.0001$) and organized sarcomeres, with a decrease in the time to peak amplitude ($p = 0.0002$ at 1 Hz) of their calcium transients [238]. Similarly, investigations of Matrigel® and fibronectin coated glass slides seeded with human embryonic stem cell (hESC)-derived CMs showed that grooves with widths between 30 μm and 80 μm enhanced CM alignment, leading to an increase in sarcomere alignment relative to the long axis of the pattern [237]. Overall, these results suggest that CM functional maturation is strongly linked to substrate properties, including stiffness and topography.

4.1.1.3. Time dependence and duration of substrate stiffness signaling: Several recent studies have demonstrated that time and duration of the mechanical stimulation can have profound impacts on construct function [215,231–233]. This is not surprising, as time dependent changes in mechanics are observed during development. In an improvement to 2D static substrate design, Young et al. evaluated the response of chick embryonic heart cells plated on substrates with elastic moduli that increase slowly over time, mimicking the stiffening of heart tissue during development [35]. Thiolated-hyaluronic acid (HA) hydrogels were crosslinked with poly(ethylene glycol) diacrylate; the modulus was controlled by changing crosslinker molecular weight [239] and cell adhesion was enabled by collagen I coating. CMs plated on the stiffening gels displayed greater maturation as measured by an increase in the sarcomere length of the cells in culture and increased expression of NKX2.5 and cardiac troponin T (cTnT) as compared to cells cultured on a static substrate modulus (PAAm gels coated in type I collagen described above, [214]) [35].

This 2D culture system showed the importance of time-dependent mechanical properties in the maturation of CMs *in vitro*, mimicking the changes observed *in vivo*. While this time-dependent system more closely replicates the mechanical properties of developing cardiac tissue, it does not account for the significant heterogeneity of proteins in the ECM that change as a function of developmental age [43], which is partially responsible for this mechanical property change. Given that composition can alter the ability of cells to sense matrix stiffness or stretch [211,240], understanding how complex composition regulates mechanical responses to substrate stiffness is a critical area for future research.

4.1.2. Step-wise or static mechanical stretch of cardiac cells—Step-wise or static stretch is shown in Fig. 2B, and is characterized by either an increase in percent stretch over time or an initial stretch following plating that is maintained over the time in culture. In key early investigations into the effect of static stretch on CMs using 20% strain, Sadoshima et al. evaluated the mechanosensitivity of isolated neonatal CMs, establishing that sarcomeric Z-disk proteins, integrins, sarcolemmal ion channels, and G-protein coupled receptors contribute to a cell's ability to sense it's environment as measured by northern blots and changes in ^{3}H-labeled amino acid uptake [149]. Additional investigations suggest that mechanical stretch is a key *in vitro* component for maintaining proper excitation-contraction coupling, demonstrated via differences in microfibrillar architecture and turnover [241,242], the activity of stretch activated ion channels [243,244], and the organization of focal adhesions [245] and overall gene expression [246] in CMs cultured with and without step-wise or static stretch. Another investigation used isolated neonatal CMs cultured on collagen and laminin coated elastic wells to determine the role of stretch and integrin binding in CM maturation using a step-wise increase in strain over four days (25%) [247]. With stretch, neonatal CMs demonstrated improved alignment, an increase total myosin heavy chain (MHC) content, an increase in the number of bi-nucleated CMs, and an increase longitudinal cell area compared to the non-stretched controls. Vandenburgh et al. hypothesized that this increase in bi-nucleation and longitudinal area is indicative of physiological hypertrophic growth and the increase in alignment and both α- and β-MHC isoform content suggest improved maturation [247]. However, it is known that MHC isoform expression changes during the course of development, and the balance shifts from one isoform to the other during development and maturation [248,249], suggesting that these changes in MHC protein expression are not enough to show improved CM maturation under these conditions. Regardless, these results were some of the first to highlight the importance of mechanical stimulation for the growth and maturation of neonatal CMs, paving the way for the future design of tissue-engineered constructs that possess functional properties similar to native myocardium.

4.1.3. Dynamic mechanical stretch of cardiac cells—Dynamic stretch (Fig. 2C) mimics the cyclic filling of the ventricles with blood during diastole making this type of mechanical stimulation more relevant to cardiac muscle as compared to step-wise stretch. Many investigations have shown that dynamic stretch affects intracellular organization, intra- and extracellular tension, FA formation, gene expression, and protein expression [63,139,140,146,150,201,208,209, 250–253]. Commercially available systems have been designed to allow for mechanical stimulation of cells plated on top of elastic substrates

(Flexcell International, Inc. [254,255]). The Flexcell systems have been utilized in a variety of CM-specific investigations, especially those that evaluate the combined role of stretch and small molecule or growth factor delivery on CM phenotype [140,256].

Mechanical stretch has a variety of effects on CM phenotype. For example, neonatal rat CMs seeded on gelatin-coated Flexcell® membranes and cultured under cyclic mechanical stretch (24 hours, 10%) [201], showed alterations in gap junction expression and organization in 2D. However, this study was unable to show that this change in gap junction expression correlated with improved CM maturation via functional measures of spontaneous beating rates or construct contractility. Additionally, mechanical stretch affects focal adhesion kinase (FAK) activation [253, 257–260], which may regulate the hypertrophic and adhesive responses of CMs *in vitro*. In one study, Leychenko et al. aimed to determine the mechanism by which stretch activates production of VEGF using a Flexcell system coated with laminin [140]. Results showed that stretch induced a 3-fold increase in VEGF secretion in adult rat CMs compared to static controls and further experimentation demonstrated that cyclic mechanical stretch activated the NF-κB, MAPK/ERK1/2, and PI3K pathways in adult rat CMs [140]. These pathways are also activated via integrin binding, but results suggested that only NF-κB activation mediates *in vitro* VEGF secretion [261]. While this mechanistic evaluation is insightful, it does not eliminate MAPK/ERK1/2 and PI3K/Akt pathways from playing a role in dynamic stretch induced cardiac hypertrophy, given that additional studies point to other mechanisms (other than VEGF secretion) that impact stretch-induced hypertrophic responses of CMs [56,209,262]. Future work should focus on systematic approaches that evaluate the role of integrin binding in hypertrophic responses via use of varying substrates, pore sizes, and stiffness, to alter FA complex organization.

4.1.3.1. Impact of mechanical stretch on hypertrophic and physiologic growth of CMs: The typical response of CMs to stretch is hypertrophy [138,263], but understanding the mechanism and the role of mechanical stimulation parameters in these pathways has proven to be a challenge. For example, angiotensin II (Ang II), when expressed under specific conditions, can induce hypertrophy in CMs [264]. Ang II expression *in vitro* can be modulated through changes to the frequency and duration mechanical stretch [49,265] via both the JNK1/2 pathway (negative) and p38MAPK pathway (positive), with long durations in mechanical stretch leading to greater Ang II expression and a reduction in JNK1/2 activation [49], suggesting that frequency and duration are critical parameters in determining CM response to dynamic stretch. However, most investigations do not evaluate changes in cycles per minute or duration of the stretch in their experimentation, leading to contradictory and confounding results. For example, another study suggested that stretch-induced hypertrophy is not a factor of paracrine signaling via Ang II, transforming growth factor (TGF)-β1, or IGF-I, and is instead a factor of the direct mechanical forces applied to the cells [263].

Similarly, PKC activation and downstream signaling has been shown to be regulated by duration of mechanical stretch. PKC is a kinase responsible for activation of a variety of hypertrophic pathways, most notably through G-protein coupled receptor activation or activation of the ERK1/2 pathway [266–268]. Using collagen I-coated Flexcell® plates, Malhotra et al. demonstrated that chronic stretch (18–20% elongation, 60 cycles/min, 48

hours) increased GRK2 activity in neonatal rat CMs, accompanied by an increase in total protein content and upregulation of atrial natriuretic factor (ANF) and β-MHC gene expression in response to stretch compared to unstretched controls [252]. These results suggested that chronic stretch at higher cycles per minute activated different pathways compared to *in vitro* experiments utilizing shorter duration mechanical stretch regimes. Future work should aim to understand the role of time and duration of the stretch signal in maturation of isolated CMs.

In another study, TGF-β signaling in embryonic mouse CMs cultured on collagen I-coated Bioflex plates exposed to cyclic stretch of 16% at 1 Hz for 24 h was reduced compared to a static control [127]. Stretch increased CM proliferation, size, cardiac gene expression, and myofibril protein levels, reduced TGF-β expression, and repressed components of the TGF-β signaling pathway, suggesting that changes in mechanical stimulation reduces TGF-β signaling. These results have implications for patients with congenital heart defects, such as HLHS because the mechanical load on developing embryonic hearts is different for these patients. Evaluation of patient data showed that expression levels of TGF-β pathway genes were lower in the right ventricles of HLHS patients compared to tissue samples from left and right ventricles of patients without heart disease [269]. Future work using dynamic stretch models should focus on studying the effects of biomechanical stimuli on normal and pathological cardiac development, to aid in designing treatment methods and strategies for young patients.

4.2. Mechanical stimulation of biomaterial constructs in 3D

Translation of 2D data to 3D constructs is not always applicable due to the variation in cytoskeletal arrangement in 2D vs. 3D [270]. In 2D, increasing the substrate stiffness leads to an increase in CM contraction force, though this can be modulated via addition of small molecules or changes in the protein-based surface coating. In 2D, stiffness and pore size are tethered, making it difficult to determine the individual roles of matrix stiffness, matrix porosity and ligand organization [214, 228–230,236,271–275]. In 3D, the role of matrix elasticity is even more elusive due to the complexity surrounding FA complex formation, integrin binding, cytoskeletal rearrangement, and availability of nutrients, increasing the difficulty in understanding the specific roles of these parameters. The variability in results is partially due to the interplay between cell-material and cell-cell interactions. To better control these parameters and create mature constructs that mimic native tissue, 3D *in vitro* systems have been developed using a variety of methodologies and materials [276–279]. Even without mechanical stimulation, 3D constructs that promote alignment of seeded cells improves CM gene and protein expression, as well as tissue function [27,280], and these results improve with the addition of mechanical stimulation. This section highlights the devices constructed to achieve alignment and maintain beating of isolated CMs or differentiated stem cells cultured in 3D and focuses on the impact mechanical stimulation has on CM phenotype and function.

A variety of bioreactors has been initially developed to cyclically stretch 3D engineered cardiac tissues [27,281–283]. One of the first bio-reactor designs to show functional improvement of cardiac tissues with stretch was developed by the Eschenhagen group,

utilizing freshly isolated neonatal rat [139,284] and chick [139] cells encapsulated within Matrigel® supplemented collagen I gels [139,284]. Using neonatal rat CMs, Zimmerman et al. generated tubular engineered tissue constructs (EHTs) that had a maximum twitch amplitude of 0.51 mN, with results positively correlated to Matrigel® concentration [284], suggesting that complex ECM composition impacts CM maturation and EHT development. In addition to demonstrating an increase in contraction force with increased time in culture, the neonatal rat CM-seeded EHTs exhibited a positive force-length and negative force-frequency relationship, suggesting that the EHTs were similar to native tissue [284]. In a similar study, EHTs formed from either neonatal rat or chick CMs were uniaxially stretched in collagen gels and, after four days, they exhibited improved CM organization, increased cell length and width, myofilament length, and mitochondrial density, as well as upregulated metabolic activity, and a 40% increase in sarcomeric α-actin [139]. Overall, these results suggest that mechanical stretch is critical for the 3D maintenance and maturation of CM phenotypes *in vitro*, yet the results do not report on the magnitude or frequency of stretch that leads to maximal twitch force. Subsequent improvements to this design, such as the formation of ring-shaped constructs enabling better control over the parameters used, demonstrated constructs with highly organized sarcomeres, as well as increased adherens and gap junction formation following seven days of culture with mechanical stimulation (10% strain, 2 Hz) [285].

In another design, collagen gels were utilized to evaluate the role of mechanical properties in contraction force development. In this design, the gels are anchored at their endpoints and both water loss and compaction of the gel by the entrapped cells enables mechanical stimulation via passive tension. Specifically, the gel was formed and suspended between two parallel PDMS posts [286,287]. The deflection of the posts allowed for the determination of force exerted by the cells in the hydrogels as the system contracted and the collagen gel was compacted. Given the limited remodeling and growth potential of isolated CMs, inclusion of isolated CFs within the construct is necessary for compaction of the biomaterial [288–290]. To create *in vitro* models of disease, stiff rods replaced the soft PDMS pillars to mimic increased afterload [291].

To expand on this design, indentation of a 3D collagen construct by an atomic force microscopy tip at varying magnitudes and frequencies enhanced the contractility of tissue engineered constructs seeded with a native myocardial population of cells (including both myocytes and CFs) in both large constructs [286] and small micro-fabricated tissues [292]. In the larger tissue constructs, ramp sizes of 1, 2, and 3 μm were used to apply a peak force between 4 and 5 μN. Even though these experiments evaluated frequencies between 0.5 and 2 Hz, and demonstrated that 2Hz resulted in enhanced CM phenotypes, the construct maintained a beating frequency near 1 Hz, suggesting that indentation could mechanically activate the cells within the construct, but not pace them. From this study, it was hypothesized that while mechanical stretch aids in maturation of neonatal CMs and improves striations and cell alignment, it does not influence calcium signaling or cellular synchronicity, which are key parameters for tissue maturation (Fig. 1A-D).

Another specific bioreactor design was created to cyclically stretch porous collagen sponges (Gelfoam®), which were glued to a petri dish on one end and attached to a coated steel bar

(used for dynamically controlling stretch) at the other [293]. In one study utilizing this system, human CMs obtained from a ventricular biopsy were seeded on top of a Gelfoam® sponge and stimulated (80 cycles/minute for 14 days) [294]. Results showed an increase in collagen matrix formation and organization as well as greater infiltration of isolated cells into the sponge in response to mechanical stretch.

4.2.1. Compressive mechanical strain and perfusion-induced strain of 3D cardiac tissues—Aside from the traditional methods of inducing mechanical stimulation via physical strain of the biomaterial construct, two other methods have been explored. First, strain can be applied in the form of compression, which, while dynamic, stimulates the cells in the opposite way native muscle stretches. Secondly, fluid shear stress can be applied to a construct via perfusion. Perfusion can be continuous, such as that applied using a steady flow rate, or cyclical, such as that applied using a peristaltic pump. The frequency of the pulsatile flow can be tuned to mimic heart rate of humans (1 Hz = 60 beats per min (bpm)) or rodents (2 Hz or 3 Hz, 120 or 180 bpm), to match the origin of the cells within the construct.

In one study, the effect of shear and compressional strain applied at either continuous or intermittent cycles on cells isolated from neonatal hearts was evaluated using RGD-grafted alginate hydrogels subjected to combined compression (1 Hz, 15% strain) and fluid shear stress [295]. Daily, short-term (30 min) compression (intermittent compression) for four days induced the formation of cardiac tissue with typical striations and increased Cx43 expression compared to the continuously compressed constructs where the cells remained spherical. In addition, secretion levels of basic fibroblast growth factor and transforming growth factor-β (TGF-β) were higher in the daily, intermittently compressed constructs, likely accounting for the change in CM phenotypes, suggesting that dynamic stimulation in an *in vitro* culture system is important for maintaining CM phenotype. However, it is important to note that TGFβ release could indicate that CFs are transitioning myofibroblasts, similar to CF activation following MI and thus longer term studies looking at CF activation and production of ECM should be performed.

Perfusion is necessary to maintain adequate nutrient supply and gaseous exchange, which is critical for generating large tissue (on the order of mm or larger) for cardiac repair [296–298]. However, pulsatile perfusion can be utilized to provide shear stress to the cells at the surface of the material or within a channeled scaffold as a means of mechanically stimulating the seeded cells [299]. Pulsatile flow at 1 Hz was compared to non-pulsatile flow using two rates: 1.50 mL/min and 0.32 mL/min. Pulsatile flow led to significantly lower excitation thresholds needed to stimulate the constructs, with higher contraction amplitudes at the higher flow rate, while pulsatile perfusion at the lower flow rate lead to the highest maximum capture rate accompanied by the highest cellular length and diameter, suggesting these cells were undergoing hypertrophy [299]. These results suggest a potentially important role for dynamic shear stress in determining CM phenotype.

More recently a novel method utilizing magnetic nanoparticles to control mechanical stimulation within 3D macroporous alginate scaffolds was described [300]. Neonatal CMs seeded within the macroporous scaffold were mechanically stimulated via an external

magnet providing an alternating magnetic field of 5 Hz. Stimulated constructs demonstrated greater amounts of anisotropically organized striated cardiac fibers compared to non-stimulated constructs, along with an increase in AKT phosphorylation (which is associated with hypertrophy [52,53,301,302]) after short-term (20 min) external magnetic field application. p38 mitogen-activated protein kinase (MAPK) activation was similar in stimulated and non-stimulated constructs [300]. While these results are interesting, further studies are necessary to optimize the material and stimulation parameters as well as to evaluate the effect of magnetic field stimulation alone on cell function.

4.3. Three-dimensional stretch-induced stem cell differentiation toward cardiomyocytes

Studies of the effects of mechanical stretch on stem cell derived CMs are less abundant and the results are contradictory. Several groups have demonstrated that either step-wise or dynamic stretch in combination with specialized differentiation medium resulted in enhanced cardiogenesis in ESCs, hiPSCs, [29,127,234,303–305] and mesenchymal stem cells (MSCs) [135,306,307]. For example, bone marrow (BM)-MSCs cultured under differentiating conditions with stretch on a silicone membrane (10% strain at a frequency of 1 Hz + 10 μM 5-azacytidine for 24 hours) demonstrated greater cTnT expression compared to BM-MSCs differentiated using only mechanical or small molecule stimulation alone [308]. However, the level of strain investigated varies widely (5-75%) and the level of cardiogenic differentiation of the stem cells was frequency [304], amplitude [135], and duration dependent [231–233].

Mechanical stimulation systems used to improve stem cell differentiation are very similar to the systems used to enhance maturation of isolated CM populations. The role of cyclic stretch on differentiation and maturation of hESC-derived CMs in 3D was evaluated by plating the cells on Gelfoam sponges (30 × 10 × 7 mm, ~12% strain, 1.25 Hz (75 cycles/min), continuous cycling with each stretch comprising 37% of the cycle duration, 7 hours, [309]). Histological analysis showed a greater amount of cTnT-expressing cells in stretched constructs and results showed a fourfold increase in initial cell seeding density enhanced cTnT expression. Stretched populations contained a higher fraction of CMs compared to support cells and a higher proportion of NKX2.5-GFP^{+}/CD90-APC – CMs compared to non-stretched scaffolds (29.8 ± 1.5% vs. 23.5 ± 0.4%, $p < 0.05$, respectively). Nkx2.5 is a transcription factor found in developing hearts, deemed necessary for proper embryonic cardiac development [168,310,311]. Results suggest that stretch maintained and enhanced viability, attachment, and continued maturation of the hESC-derived CMs within the Gelfoam scaffold [309]. Similar results using murine ESC-derived CMs seeded within collagen I-fibronectin hydrogels showed that 10% strain applied at 3 Hz for days 7-10 of 3D culture resulted in significantly greater gene expression of α-cardiac actin, α-skeletal actin, α-MHC, and β-MHC compared to constructs that received 10% strain at only 1 Hz [304].

In another study using hESC-derived CMs and hiPSC-derived CMs in collagen gels, mechanical load and vascular cell co-culture (addition of stromal or endothelial cells) improved CM proliferation [29]. Further evaluation demonstrated that four days of cyclic stress conditioning increased hypertrophy via significant upregulation of gene expression of hypertrophic genes in hiPSC-derived CMs (β-MHC, cTnT, ANP, L-type calcium channel

subunit 1Cα, sarcoplasmic calcium channel/ryanodine receptor, and SERCA2). Four days of mechanical strain (either static or cyclic stretch) also improved alignment and striations within the constructs [29]. One important aspect of the investigations reviewed here is that, many of the experiments described within this body of work demonstrated that static and cyclic mechanical stretch yielded statistically similar results, while both methods were significantly different from 2D culture. The similarity in response to varying stimulation regimes suggests that additional factors such as small molecule delivery, cell culture medium composition, time allowed for differentiation prior to stimulation, and overall duration of the experiment may be critical factors in cell response, in addition to mechanical stimulation. The complex sets of variable utilized in these experiments makes it difficult to pinpoint the optimal stimulation regime for generating mature cardiac tissue constructs.

4.4. Future goals and ongoing improvements

While these studies have established the significant effect of mechanical stimuli on CM function and differentiation of stem cells to CMs, some questions remain. One particular question is the role of temporal variation of stimuli. All of these studies conducted mechanical stimulation at a constant amplitude and frequency, while the mechanical stimulation *in vivo* is highly variable. Since variability is an essential component of healthy heart function [312], the introduction of variability in mechanical stimulation may improve the maturation of CMs within a 3D construct. Isolated neonatal CMs cultured in a dynamic bio-reactor were used to evaluate variable frequency of mechanical stimulation in a cycle-by-cycle manner (see the mechanical descriptions in Fig. 4E) [313]. Gaussian stimulation may promote physiological over pathological hypertrophy as shown by lower ratios phosphorylated ERK1/2 to ERK1/2. Activation, or phosphorylation or ERK1/2 activates the MAPK/ERK pathway, which has been implicated in pathological hypertrophy [33]. Further work should be carried out to understand how variability in mechanical stimulation can promote physiological over pathological hypertrophy.

5. Electrical stimulation of cardiac constructs

Excitation–contraction coupling is critical for heart development and function and electrical stimulation can be used to induce synchronous contractions within cardiac constructs. Electrical stimulation affects the rate, duration, and number of action potentials within CMs, which increases the percentage of spontaneously beating cells and aids in cell synchronization and calcium handling within a construct. Promoting synchronous contractions improves tissue homogeneity and interconnectivity, which ultimately leads to greater contraction force generation by the construct [176,314]. Many protocols have been developed to electrically stimulate cardiac, stem, and progenitor cells with positive results in both 2D and 3D formats (see Tandon et al. for an example protocol [315]). Most electrical stimulation setups are simple in design, using two electrodes to provide bulk field stimulation to the culture container with electrical impulses directed through the culture media. Electrodes are often either made of carbon rods or platinum wires and optimization of the stimulation duration, voltage, and frequency improves cell response in a given biomaterial system [316].

5.1. Electrical stimulation and cardiac hypertrophy in two dimensional cultures

Electrical stimulation of cells in culture has been used to mature isolated muscle cells for over 40 years [317,318] and has been shown to activate many pathways leading to changes in a variety of intracellular activities such as transcription factor activation, calcium handling, response to oxidative stress, protein kinase expression and activation, and phosphatase activation. For example, transcription factors such as NFAT3, GATA4, NRF-1 (nuclear respiratory factor 1), c-Jun, and cyto-chrome C are important for processes related to growth and maturation of CMs, mitochondrial proliferation within CMs, and differentiation of progenitor cells and the expression levels are upregulated either transiently or permanently by electrical stimulation [319,320]. Calcium handling and oxidative stress are modulated in electrically stimulated CMs, through activation of the CaMK (Ca^{2+}/calmodulin-dependent protein kinase) pathway [319,321,322]. Activation of CaMK-I and CaMK-IV leads to activation of phosphatases, such as calcineurin, which induce hypertrophic responses *in vitro* and overexpression *in vivo* leads to pathological hypertrophy [321,322]. These results demonstrate the need for greater understanding of the duration of electrical signaling that results in pathological verses physiological response so that *in vitro* culture methods can be clearly designed to promote physiologic or pathologic responses, as desired by the research aims.

As shown in Fig. 1B, the connection between calcium handling and action potentials with CM contraction are critical for the maturation of CMs. The connection between contraction and action potential generation are controlled by gene expression and protein expression of mature CM markers (MHCs, troponins, Cxs) and ion channels. Not only are expression levels of these key proteins controlled via mechanotransduction pathways as discussed in the previous section, but they are also regulated via electrical stimulation. For example, cells plated on tissue culture plastic and stimulated (10 Hz, 1 V, 5 ms) for six days showed significantly greater alignment, expression of Cx43, increased ratio between cell length and cell width compared to non-stimulated samples [323]. In addition, Kcnh2 and Kcnd2, which are voltage-gated potassium channels related to the human ether-ago-go gene (HERG), were upregulated in stimulated culture compared to the control. HERG is required for the final repolarization of the ventricular action potential and mutations in this gene lead to long QT syndrome and fatal arrhythmias [172,324–326], demonstrating the importance of these signaling pathways in development and CM maturation. Results suggest that electrical stimulation can improve the maturation of fetal CMs *in vitro* and parameters such as frequency and voltage impact CM behavior.

5.1.1. Electrical stimulation platforms for the development of cardiac platforms for drug testing—

One way to translate *in vitro* results to clinical applications is through the development of culture platforms that mimic native function in a high-throughput, miniaturized platform using tools such as microfluidic channels, micro-devices, and chips for testing the safety and efficacy of drugs via CM response [327–333]. Microscale platforms are beneficial due to the small size, which enables high throughput analyses, utilization of limited cell numbers, and adequate oxygen and nutrient delivery, enabling analysis of cellular response to various drug formulations as well as concentrations without draining resources. *Au* et al. developed a cell culture chip with microgrooves and

microridges of precisely defined depth, width, and periodicity to investigate how CMs responded to the patterned surfaces as compared to smooth controls [329]. Two gold electrodes (1 cm spacing) were placed either parallel or perpendicular to the grooves and, after seven days of culture and stimulation, neonatal CMs were contractile and aligned along the microgrooves, as evidenced by sarcomeric α-actinin staining (the best responses were at a periodicity of 1 μm). Biphasic electrical stimulation resulted in Cx43 expression localization near cell-cell junctions as opposed to the punctate expression in unstimulated neonatal CMs and microgrooves parallel to the electric field led to increased elongation compared to electrical stimulation delivered perpendicular to the grooves [329], suggesting that both topographical structure and electrical field stimulation affect CM response in small microdevices. In another study, a heart-on-a-chip was designed for high throughput analysis of CM function in response to varying matrix properties (alignment) or small molecule concentrations (isoproterenol) [330]. Neonatal CMs were seeded onto the chip and cantilever deformation was observed (2 Hz with 10–15 V of a bipolar square pulse of 10 ms duration). The design allows for continuous measurements of both diastolic and systolic stresses as well as twitch force generation without sacrificing the sample [330]. This heart-on-a-chip device has been applied to many applications including the development of disease models (e.g., Barth Syndrome [334]) and drug testing [335–337], with future applications for stem cell differentiation and co-culture systems [338].

5.2. Electrical stimulation of biomaterial constructs in 3D

Electrical stimulation of a diverse array of 3-D constructs created using a variety of different biomaterials has led to the development of bioreactors and stimulation regimes which assist in stem cell differentiation towards a myocyte phenotype as well as improved cardiac cell function *in vitro* [184,315,316,339–344]. Many biomaterials commonly used for *in vitro* cell culture are not electrically conductive, thus necessitating external methods for achieving synchronous contraction across a 3D tissue. Additional efforts have focused on making scaffolds electrically conductive via additives, such as carbon nanotubes and graphene (See [345–349]).

Radisic et al. established the role of electrical stimulation in CM maturation and tissue coordination using isolates of CMs and CFs from neonatal rats seeded in Matrigel® onto collagen sponges [314]. After three days in culture, constructs were continuously stimulated for five days via electrical pulses (rectangular, 2 ms, 5 V/cm, 1 Hz) that mimic the native myocardium [350]. Electrical stimulation led to continual development of conductive and contractile properties in cardiac constructs, measured by elevated levels of MHC, Cx43, creatine kinase-MM, and cTnI, significantly greater contraction force, and a significant increase in the maximum pacing frequency that maintains synchronous construct contractions (maximum capture rate) [314]. Conversely, using a blocker of L-type calcium channels [350], gap junctions [351], and PI3K pathway (cytoskeletal rearrangement [352]), the study confirmed that functional gap junctions, cytoskeletal organization, and excitation–contraction coupling are all necessary for construct contractility [314]. Furthermore, stimulation before day three in culture prevented intracellular accumulation of necessary cardiac proteins, limiting the resulting CM maturation and contractility [353], establishing the need for precise control over the stimulation regime and an understanding of CM

phenotype prior, during, and after stimulation. In another study, 3D tissues were formed by layering 2D cell sheets show greater conduction-contraction integration between the layers following only one day of electrical stimulation [354], showing that addition of electrical stimulation can assist in creating synchronous constructs from asynchronous cell populations though the development of CM coupling.

Electrical stimulation has also been evaluated in combination with other cues, such as small molecule delivery, changes in integrin binding, or combined use of architectural alignment and growth factor delivery in conjunction with electrical stimulation. For example, IGF-1 is known to protect CMs from hypertrophic and oxidative stresses and promote survival following injury [53,343,355–357]. In aligned PGS scaffolds seeded with neonatal CMs, addition of IGF-1 in combination with electrical stimulation (2 ms in duration, 5 V/cm amplitude, 1 Hz frequency) significantly increased cell diameter and qualitatively enhanced Cx43 expression over eight days in 3D culture when compared to either electrical stimulation or IGF-1 addition alone [343]. However, other measures, such as the excitation threshold and cTnT expression were statistically similar across all combinations of electrical stimulation and/ or IGF-1.

5.3. Electrical stimulation and stem cell differentiation

As suggested in Fig. 1B, action potentials and calcium handling are directly related to contraction and therefore, generation of a synchronously contracting construct requires intercellular communication, as shown by its role in stem cell differentiation and CM maturation [344, 358,359]. Many investigators have utilized electrical cues to promote intercellular communication and CM maturation. For example, embryoid bodies were cultured under electrical stimulation (6.6 V/cm, 1 Hz, and 2 ms pulses) for four days and results showed upregulation of HCN1 (potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1), MLC2v (myosin light chain 2v), SCN5A (sodium channel, voltage gated, type V alpha subunit), SERCA, Kv4.3 (potassium voltage-gated channel subfamily D member 3, outward potassium current), and GATA4 gene expression [360]. In addition, electrical stimulation increased cellular elongation, increased proportion of cTNT positive cells and an increased proportion of cells exhibiting lengthening of their action potential duration. In another study, Nunes et al. developed a cardiac biowire platform where a cell-laden collagen I gel compacts around a suture, forming a wire like structure [176]. This cardiac biowire platform was designed to enhance maturation of hiPSC-derived CMs using tunable electrical stimulation (0, 3, or 6 Hz). Stimulated hiPSC biowire constructs showed increased myofibril ultra-structural organization (demonstrated by α-actinin and cTnT staining and imaging of desmosomes) increased conduction velocity and maximum capture rate, along with decreased excitation threshold and improved electrophysiological and Ca^{2+} handling properties as shown by response to epinephrine [176]. In another study, a bioreactor was designed to deliver either monophasic (5 V for 2 ms, 1 Hz) or biphasic (+2.5 V for 1 ms, −2.5 V for 1 ms, 1 Hz) electrical pulses with tunable amplitude, pulse width, and frequency to human cardiac progenitors cultured on gelatin coated glass slides [344,361]. After 72 hours, biphasic electrical stimulation resulted in upregulation of early cardiac transcription factors (MEF2D, GATA-4, Nkx2.5), greater expression of late cardiac sarcomeric proteins (cTnT, cardiac α-actinin, and SERCA2a), and

earlier expression of Cx43 compared to monophasic electrical stimulation [344]. Taken together, these results demonstrated that biphasic stimulation achieved cardiac differentiation more effectively than monophasic electrical stimulation. Evaluation of differentiation of mouse embryonic stem cells over time also showed that cells at different stages of differentiation had different responses to electrical stimulation, as measured by upregulation of cTnT and β-MHC and downregulation of Nanog [358]. While the results were inconsistent, they did show that the point in the differentiation process that electrical stimulation was applied did enhance final cell responses (intermediate and terminal electrical stimulation had more of an effect than early stimulation). In addition, amplitude played a role in the degree to which the changes in protein expression in stimulated cultures varied from non-stimulated cultures [358]. In another study, Hirt et al. demonstrated that hiPSC-derived CMs cultured within fibrin-Matrigel® composite constructs stimulated at 2 Hz in the first week and 1.5 Hz in the second week developed 1.5 fold higher forces than unstimulated constructs after two weeks [362]. Electrical stimulation improved alignment and organization within the hiPSC-derived CM constructs, suggesting that continuous pacing improved structural and functional properties of engineered tissues.

While these studies demonstrate the critical importance of electrical stimulation in differentiation of stem cells, the work presented does not describe the mechanism by which electrical stimulation produces these benefits. Generally, electrical stimulation has been shown to induce intracellular reactive oxygen species (ROS) generation *in vitro* and this may be one mechanism by which electrical stimulation induces differentiation [363–365]. To evaluate the role of intracellular ROS generation resulting from electrical stimulation on differentiation of hESCs into CMs, an electrical stimulation chamber was constructed from PDMS for the culture of embryoid bodies to evaluate spontaneous contractions, expression of cTnT, and sarcomeric organization [359]. Experimental parameters that result in the highest ROS generation were determined by altering the electrode material, stimulus length (1 s and 90 s) and the age of embryoid bodies at the start of electrical stimulation (4 and 8 days) and the highest rate of ROS generation was observed for 90 s stimulation using stainless steel electrodes. Results demonstrate that the method of electrical stimulation, coupled with the biological parameters within the system may initiate secondary interactions that may lead to confounding results. Future work in this area should aim to optimize important electrical stimulation parameters including amplitude, duration, and timing to achieve more mature and functional CMs for *in vitro* use.

6. Combined stimulation of biomaterial constructs in 3D

As described in the sections above, mechanical or electrical stimulation can individually impact growth and maturation of CMs cultured *in vitro*. Cell-cell coupling of myocytes in the heart enables the propagation of electrical signals to produce the almost synchronous contractions of the whole organ to adequately pump blood to the body [366]. The coupling of electrical excitation and mechanical contraction is essential for proper organ function (Fig. 1B). As such, evaluating the role of physiologically relevant combined electrical and mechanical stimulation in 3D culture is of critical impact to the design of tissues, patches, and constructs for clinical application. In most cases, two main types of mechanical stimulation are utilized: 1) Mechanical force via flow, whether continuous or pulsatile, and

2) Mechanical stimulation via physical deformation of the 3D construct, mimicking the filling of the ventricles with blood. Perfusion, or force applied to a system via flow, does not always lead to strain-based deformation of 3D scaffolds. However, dynamic or pulsatile flow systems affect downstream signaling [367,368] resulting in similar changes to gene expression and cell behavior to those measured in physical deformation systems, justifying the inclusion of flow systems as combined electromechanical systems [369].

6.1. Perfused and electrically stimulated 3D constructs

To improve nutrient and small molecule delivery to electrically simulated tissues and provide flow-based mechanical stimulation, bio-reactors have been designed which allow for both media perfusion and electrical stimulation. Aligned scaffolds containing neonatal rat CMs formed from PGS and coated with laminin showed significantly improved functional properties (increase in contraction amplitude) and cTnT expression when cultured with both perfusion (peristaltic pump, 18 μl/min) and electrical stimulation (3 V/cm, 3 Hz, monophasic square wave) compared to either condition alone or in static culture [341]. These results suggest that the improvements seen were not just due to the addition of improved oxygen and nutrient supply afforded by perfusion of the construct. Similarly, isolated neonatal rat CM maturation, as measured by increased elongation and striation and increased expression Cx43, was enhanced in perfused 3D alginate/Matrigel®-based constructs cultured under field stimulation (74.4 mA/cm^2, 2 ms, bipolar, 1 Hz) after four days as compared to perfused, but not electrically simulated, constructs [339].

Another system developed to enable perfusion and electrical stimulation in 3D is the use of a re-cellularized decellularized heart [370–372]. For example, in a decellularized rat heart re-cellularized with isolated CMs, perfusion of the heart (in at the left atrium and out through the aortic valve) was used to inflate and deflate the ventricles providing mechanical stimulation via both flow and mechanical stretch [370]. A loop was attached to the aorta to allow for physiological coronary perfusion and electrical stimulation (1 Hz, 5–20 V, 2 ms, platinum wire attached to the epicardial surface near the apex of the LV) was applied to promote cell-cell coupling. Eight days post re-cellularization, repeated pacing (<4 Hz) led to a constant systolic pressure of ~2.4 mmHg (~2% of adult rat heart function and ~25% of 16-week fetal human heart function [373]) while stimulation above 4 Hz led to a decrease in contraction pressure. This work demonstrated the feasibility of decellularizing the whole heart and using it to create *in vitro* models of a functioning heart and the variability in recellularization potential due to external cues.

In another study, the biowire platform [174,176] was improved to allow for both electrical stimulation and perfusion of wire-based constructs that mimic the cardiac bundle found in native tissue (Fig. 3). The perfusion system allowed for the delivery of different small molecules, such as nitric oxide, demonstrating the applicability of this system as a drug-testing platform. Electrical stimulation (biphasic, rectangular, 1 ms duration, 1.2 Hz, 3.5-4 V/cm) of neonatal rat CMs increased the Cx43 positive area over cTnT positive area in images taken after four days of stimulated culture compared to non-stimulated controls. In addition, results demonstrate that the orientation of CM alignment and the direction of

electrical field stimulation affects functional outputs such as excitation threshold and maximum capture rate [174], indicating the complexity in combined stimulation systems.

6.2. Bioreactors with physiologically relevant electrical stimulation and mechanical stretch

In an effort to more closely mimic the native physiology of cardiac tissue, innovative bioreactor designs have been developed that aim to incorporate mechanical stretch and electrical stimulation into 3D scaffold or hydrogel cultures [34,342,374–377]. The main goal is to synchronize contraction with appropriately timed mechanical stimulation to mimic ventricular filling, which is crucial in the design of a system that recapitulates native function. In all cases, electrical stimulation is achieved via field stimulation of the entire construct. In the reactors described here, mechanical stimulation is controlled by physically stretching the construct to mimic the stain applied to cardiac muscle when the ventricles fill with blood during a normal cardiac cycle. One of the earliest descriptions of a tunable electro-mechanical bioreactor system included the ability to stimulate constructs with a variety of unidirectional strains, demonstrating the feasibility and tunability of the platform [374]. Improvements in and experimentation with this first bioreactor have been made in a variety of ways, including increasing the number of testable samples and improving the control over the stimulation regimes. Morgan et al. designed individual bioreactor units (Fig. 4, [33,34]) based on a previous design for mechanical stimulation [27,34,375]. Electrical stimulation is separately controlled and achieved by the addition of two carbon rods to the media surrounding the construct. This combined system delivers physiologically relevant timing of combined electrical and mechanical stimulation, mimicking isovolumetric contraction (Fig. 4) [34]. Neonatal CMs cultured in fibrin hydrogels and stimulated via a “delayed” mechanism (electrical stimulation 0.49s after the start of the mechanical stimulation) that mimicked the isovolumic contraction time showed statistically greater SERCA2a expression compared to all conditions and greater Akt1 expression compared to static culture, mechanical stimulation only, and electrical stimulation only [34]. Subsequent studies also demonstrated that further alterations to the timing that offset the electrical and mechanical stimulation in non-physiological regimes led to a reduction in force generation and relaxation rates indicating that this type of system may be useful in studying functional changes to cardiac muscle in cases of arrhythmic diseases [33]. This fine control over the coordination between the signals could allow for modeling of disease progression via changes in the patterns and duration of the combined signaling milieu.

Miklas et al. built an electromechanical bioreactor platform that contains eight microtissues and two stimulating electrodes, so that each electrode pair stimulates four constructs at one time, providing greater high-throughput compared to previous designs [376]. For these microtissues, static stress is applied using a pneumatically driven stretch device. A pair of PDMS posts inside each microwell chamber allows for the calculation and verification of the stretch applied to each tissue. Tissues cultured under a static 5% strain and 3–4 V cm^{-1}, 1 Hz electrical stimulation had enhanced sarcomere alignment (cTnT) and expression of gap junction proteins (Cx43) after three days compared to either mechanical or electrical stimulation alone [376].

Another electromechanical platform developed by Wang et al. contains tissue culture chamber that can house two to four pieces of tissue construct (20 mm × 20 mm × ~3 mm) where the frequency and amplitude of the cyclic stretches and electrical pulses can be tuned to match native tissue [342]. To demonstrate the utility of this design, sheets of decellularized porcine myocardium were clamped into the reactor and injected with MSCs. Cardia differentiation of the MSCs was promoted using a 5-azacytidine-based differentiation protocol for 24 hours, followed by electromechanical stimulation and the resulting differentiation and re-cellularization of the porcine matrix was analyzed. Immunofluorescence imaging shows positive staining for sarcomeric α-actinin, MHC, cTnT, Cx43, and N-cadherin after two days of mechanical (20% strain) and electrical stimulation (5 V, 1 Hz).

7. Conclusions

Understanding the role of electromechanical stimulation on developmental pathways that lead to fully functioning cardiac tissue combined with innovative reactor designs has significantly advanced the field of *in vitro* culture of 3D tissue constructs. Current utilization of these innovative designs focuses on three main areas: disease modeling, drug testing, and critically sized construct formation. While much of the literature reviewed above has made significant strides in our understanding of the role of biophysical stimulation in the context of cardiac tissue engineering, there remain areas for further exploration, especially when considering the use of these bioreactor systems to model disease and generate *in vivo* tissue constructs.

First, when bioreactors are utilized to precondition tissue constructs prior to implantation in the development of skeletal muscle constructs, improved integration and functional repair is seen [378], suggesting that preconditioning of cardiac patches should positively impact *in vivo* functional results in cardiac repair. Current *in vivo* cellularized cardiac patches have shown limited functional benefits in comparison to the cell-free patch, suggesting that the delivered cells are not integrating well with host tissue and contributing to native cardiac muscle contraction [23]. However, changes in gene and protein expression within the constructs and host tissue suggest that delivery of cells has some benefits to the wound healing process. To further this line of research, the field of cardiac tissue engineering and bioreactor design should focus on developing preconditioning methods that achieve maturation and either physiological or pathological hypertrophy (in the context of disease models) within constructs, paying specific attention to methods to distinguish these two mechanisms. For example, to mimic physiological hypertrophy, methods to exercise constructs should be created, with a focus on varying CM phenotype based on the stimulation regime. Then, these preconditioned scaffolds should be implanted and the changes to cardiac repair should be assessed.

Second, given that the prevalence of heart disease and secondary diseases is on the rise and that mortality rates increase when patients are diagnosed with multiple diseases [1,379], *in vitro* model systems of cardiac disease should be utilized to better understand the influence of secondary diseases and their treatments on cardiovascular function. To enable these types of investigations, sophisticated bioreactors are necessary; development of bioreactors that

can mimic different disease states via control over both electrical and mechanical stimulation and particularly their timing, which can be altered in disease pathologies such as diabetic cardiomyopathy, are required. Current research has not lead to the development of fully functional constructs that mimic *in vivo* tissue behavior, suggesting that improvements in construct development and bioreactor stimulation regimes are still necessary to achieve functional engineered tissue construct for drug testing and appropriate disease models. In addition, future work should aim to use these models in combination with whole system lab-on-a-chip platforms [36] to evaluate the safety and efficacy of combined drug treatments and the role that administration of pharmaceutical regiments for both primary and secondary diagnoses has on the progression of heart failure.

Finally, one major improvement that can be made in the context of *in vitro* studies is the development of experiments that highlight the importance of *in vivo* variability and irregularity. Given that heart rate and diastolic blood pressure are variable on a beat-to-beat basis in most individuals, future work should aim to understand the role variability in amplitude and rate plays in maintaining tissue function. In humans, loss of the variability on a beat-to-beat basis is a hallmark of heart failure [312,380], necessitating further understanding of disease pathologies and better strategies for the design of *in vitro* models that adequately mimic healthy or diseased tissue. Moreover, variability in stretch amplitude has demonstrated beneficial effects in other *in vitro* culture systems where *in vivo* dynamics are variable, such as the lung [381]. Many of the reactor designs reviewed here should be able to accommodate variability in both rate and amplitude through modifications to the programs controlling construct stimulation without any major modifications to the bioreactors themselves.

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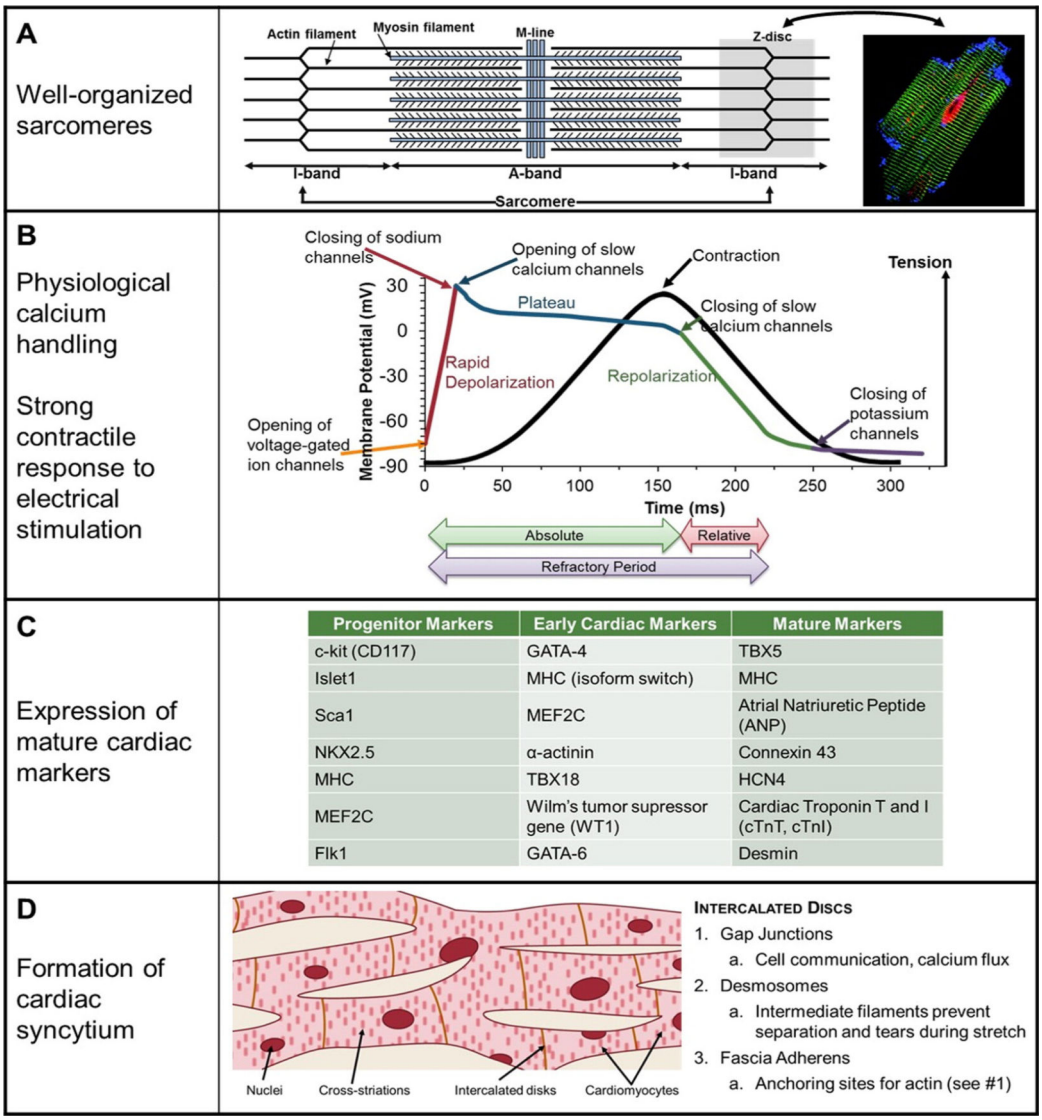

Progenitor Markers	Early Cardiac Markers	Mature Markers
c-kit (CD117)	GATA-4	TBX5
Islet1	MHC (isoform switch)	MHC
Sca1	MEF2C	Atrial Natriuretic Peptide (ANP)
NKX2.5	α-actinin	Connexin 43
MHC	TBX18	HCN4
MEF2C	Wilm's tumor supressor gene (WT1)	Cardiac Troponin T and I (cTnT, cTnI)
Flk1	GATA-6	Desmin

Fig. 1.

Useful markers of mature cardiomyocytes (CMs). A) Well-organized sarcomere structures are important for contractile phenotypes. These organized muscle structures connect to the intercalated discs via fascia adherens, which bind actin filaments. The z-disk in the sarcomere can be labeled with sarcomeric α-actinin (green) [382]. B) Physiological calcium handling is necessary for CM function. Activation of the action potential coordinates with the contractile apparatus, which can be manipulated via electrical stimulation of cultured CMs. In a bio-reactor, the contractile force or twitch force of a cell-seeded biomaterial is a measure of CM maturation over time. C) Expression of CM specific markers (gene or protein) can be used to determine if a new biomaterial, culture condition, or stimulation regime promotes greater maturation in comparison to a control or static culture. D) Formation of the cardiac syncytium is necessary for coordinated contraction of the organ. Unlike most contractile muscle, cardiac tissue is not multinucleated so signal propagation is achieved via intercalated discs. The intercalated disc is necessary for the propagation of

action potentials from one muscle fiber to another. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

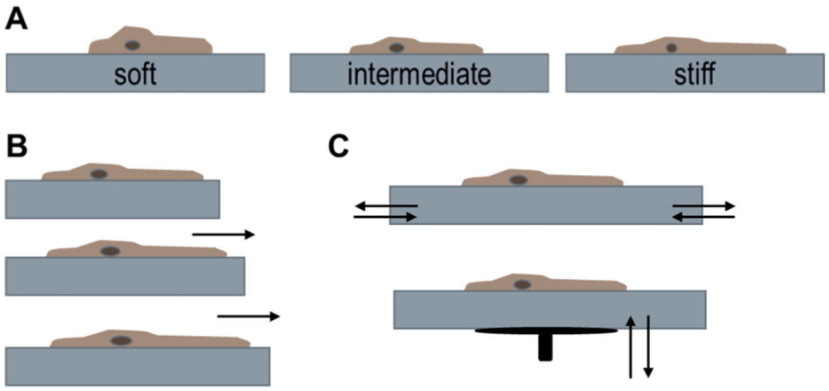

Fig. 2.

Mechanisms affecting cellular mechanics in 2D. A) Altering mechanical properties of the substrate can affect intracellular tension and ultimately gene expression and cell phenotype. B) Stepwise increase in strain applied to the material. C) Dynamic stretch of the constructs mimics the filling of the ventricles.

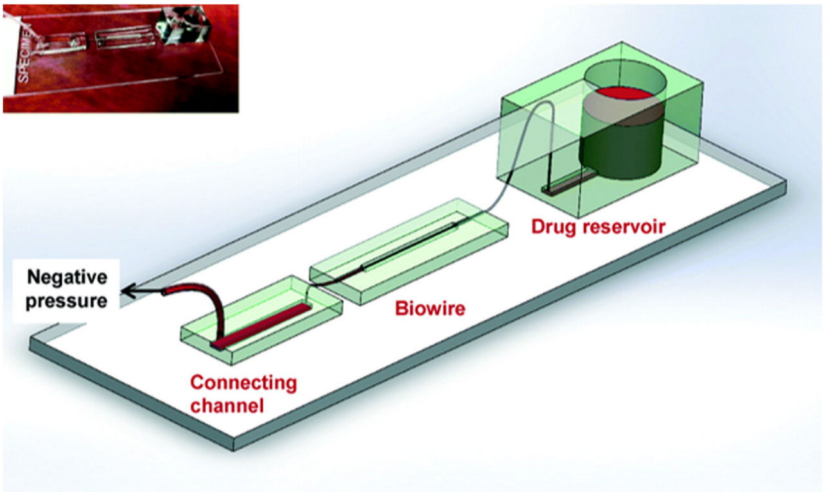

Fig. 3.

Perfusable cardiac biowire system developed in the Radisic Group. The biowire system is designed such that a gel is formed around a tubing template. The gel compacts around the tube, creating a perfusable wire. The bioreactor system consists of two microfabricated modules: 1) a drug reservoir and 2) a channel for connection of the biowire to an external negative pressure source. The biowire perfusion system was mounted on a glass slide as shown in the top-left corner [174,176].

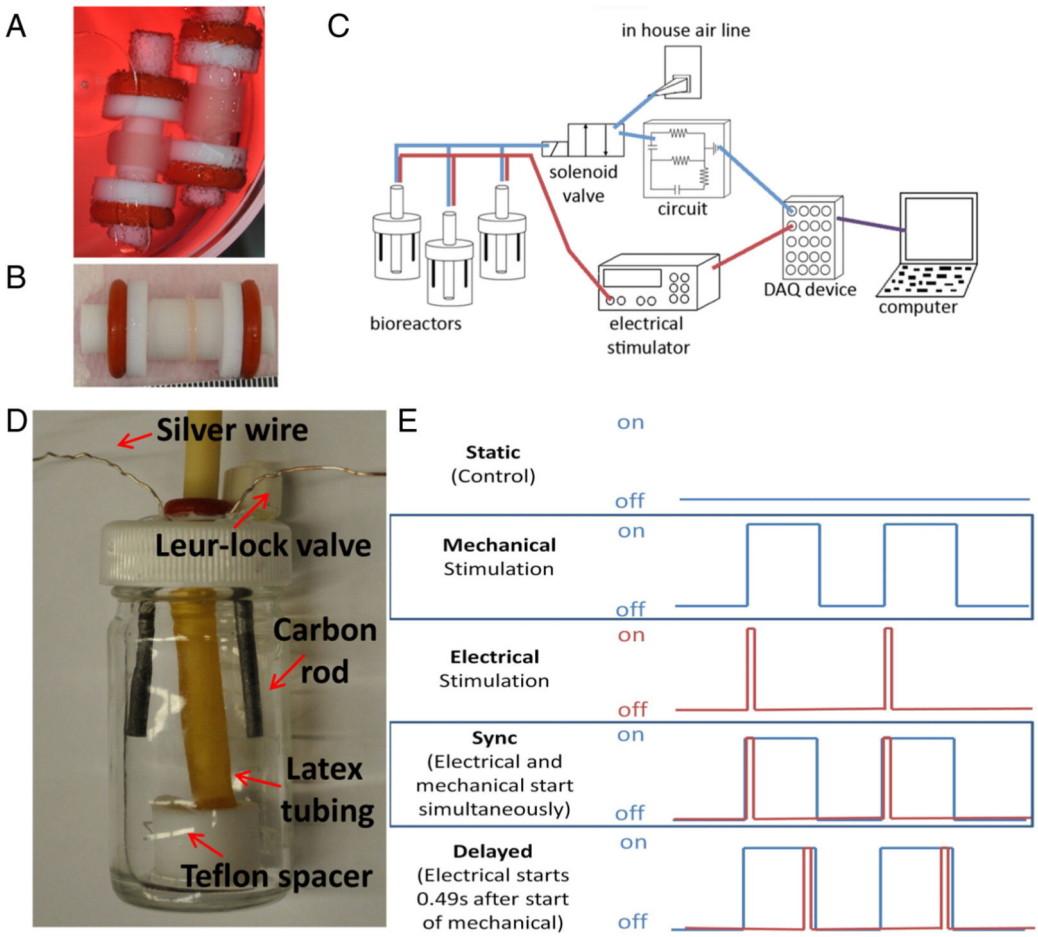

Fig. 4.

Electromechanical bioreactor developed in the Black Laboratory [34]. Fibrin constructs formed around Teflon molds on day 1 (A) and day 14 (B). C) Schematic of the bioreactor control system. A solenoid valve is connected to the compressed air line to enable the latex tube to expand during mechanical stimulation. The carbon rods are connected to an electrical stimulator and the entire custom-made bioreactor is shown in (D), with individual components labeled. (E) A diagram demonstrating the stimulation regimens implemented. The blue line represents the mechanical stretch while the red line represents the electrical stimulation. The design of the reactor control system enables the development of electrical and mechanical stimulation regimes that can mimic both healthy function (isovolumic contraction) as well as characteristic changes in disease. Control of stimulation regimes also enables the integration of construct “exercise” to replicate changes in heart rate [33,34,313,383].