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The role of tissue engineering and biomaterials in cardiac regenerative medicine

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Abstract

In recent years, the development of three-dimensional engineered heart tissue (EHT) has made large strides forward due to advances in stem cell biology, materials science, pre-vascularization strategies and nanotechnology. As a result, the role of tissue engineering in cardiac regenerative medicine has become multi-faceted as new applications become feasible. Cardiac tissue engineering has long been established to have the potential to partially or fully restore cardiac function following cardiac injury. However, EHTs may also serve as surrogate human cardiac tissue for drug-related toxicity screening. Cardiotoxicity remains a major cause of drug withdrawal in the pharmaceutical industry. Unsafe drugs reach the market because pre-clinical evaluation is insufficient to weed out cardiotoxic drugs in all their forms. Bioengineering methods could provide functional and mature human myocardial tissues, i.e. physiologically relevant platforms, for screening the cardiotoxic effects of pharmaceutical agents and facilitate the discovery of new therapeutic agents. Finally, advances in induced pluripotent stem cells have made patient-specific EHTs possible, which opens up the possibility of personalized medicine. Herein, we give an overview of the present state of the art in cardiac tissue engineering, the challenges to the field and future perspectives.

Keywords

Engineered heart tissue; hPSC-derived cardiomyocytes; cardiac functional restoration; drug screening and discovery; personalized medicine

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1. Introduction

Cardiovascular disease is responsible for greater mortality than all cancers combined in the Western world (1). Myocardial infarction (MI) causes irreversible damage to the myocardium, because the adult heart has minimal intrinsic ability to regenerate lost cardiomyocytes (CMs). After the initial insult, fibroblasts (FBs) and endothelial cells (ECs) form a dense collagenous scar that maintains wall structure but is inflexible and non-contractile, often leading to heart failure (2). The most effective present therapy to restore heart function, cardiac transplantation, is limited by insufficient availability of donor organs and the requirement for life-long immunosuppression. Left ventricular assist devices require invasive surgeries and long-term anti-coagulation.

Cardiotoxicity is a major concern for the pharmaceutical industry since differences in the electrophysiological properties of animal and human CMs limit the relevance of pre-clinical animal studies (3). Additionally, human clinical trials are limited by small sample pools and at times skewed genetic and phenotypic diversity.

Cardiac tissue engineering—based on human CMs, biomimetic scaffolds and integrated bioengineering concepts—possesses the potential to partially or fully restore cardiac function and serve as a surrogate human cardiac tissue for drug toxicity screening and personalized medicine. However, there are still many challenges to be overcome before these techniques can move toward clinical applications. This paper aims to review the present state of the art, challenges to the field and future perspectives. We will focus on tissue engineering methods that provide means of constructing human tissues for *in vitro* modelling of disease and drug discovery as well as functional cardiac patches for restoration of contractile function *in vivo* (Figure 1).

2. Cell source considerations

The objective of cardiac regenerative medicine is to repopulate the injured site with functional cells to replenish the lost cells and regenerate the damaged cardiac tissue. However, adult CMs are terminally differentiated and have a minute capacity for expansion *in vitro* from biopsies of patient's heart tissue. Therefore, alternative cell sources with abundant availability are necessary. The discovery of human induced pluripotent stem cells (hiPSCs) (4) has enabled the generation of potentially unlimited numbers of autologous CMs (5) for cell therapy and for the development of personalized drug therapies, without the ethical concerns raised by the use of human embryonic stem cells (hESCs). iPSC-derived CMs (iPSC-CMs) are additionally attractive because they can recapitulate some genetic cardiac disorders in standard monolayer cultures (e.g. Long Q-T syndrome (6)) and can also potentially be used to assess patient-specific responses to drugs prior to their use in the body. CM differentiation protocols rely on timed application of growth factors or small molecules that modulate pathways important for cardiogenesis during embryonic development. These molecules are applied to iPSCs or ESCs grown in embryoid body format (7, 8) or in monolayers (9).

In recent years, strong evidence of hESC-CM integration into the recipient heart has been found (10). Most often, *in vivo* integration of hESC-CMs into the recipient hearts has been studied using rodent models (11–13), often criticized as unsuitable due to the large difference in the heart rate between human ventricular CMs (60–120 bpm) and rodent ventricular CMs (350–600 bpm). Studies in a more comparable guinea pig model (200–250 bpm) (14) and recent non-human primate model (100–130 bpm) (15) were able to demonstrate conclusively that hESC-CMs can electrically couple with the recipient hearts post-MI, remuscularize the heart tissue (Figure 2A) and induce ingrowth of perfusable blood vessels (Figure 2B). However, the primate study indicated transient occurrences of disturbances in the heart rhythm such as: ventricular tachycardia (Figure 2C), accelerated idioventricular rhythm (Figure 2D), non-sustained ventricular tachycardia (Figure 2E) and non-sustained accelerated idioventricular rhythm (Figure 2F). These recent findings have motivated the development of new and improved approaches for selecting CMs of an appropriate maturity level in hopes of improving graft-host coupling, and the development of safe and effective methods for delivering the cells to the heart using biomaterials (15) and engineered tissues (16). Additionally, hESC-CMs are allogeneic, thus they could give rise to an immune response upon *in vivo* application; and although unlikely, the presence of residual undifferentiated cells could give rise to the formation of undesired tissue structures in the recipient hearts. Therefore, hESC- and iPSC-CMs have not progressed towards clinical trials yet.

Instead, a large number of current clinical trials focus on cell replacement through the application of bone marrow mesenchymal stem cells (17, 18), mononuclear cells (18–21) and more recently, cardiosphere-derived cardiac progenitor cells (CADUCEUS (22)). Although most of these cell types have no intrinsic ability to give rise to large numbers of beating CMs *in vitro*, they improve function *in vivo* mostly through paracrine effects as delineated in mechanistic pre-clinical studies (23, 24).

Despite showing improvements in cardiac function in both pre-clinical and clinical studies, the wide range of tested cell injection strategies (25–32) have been plagued by excessive cell death after delivery (33) and challenges with functional integration (34–36), motivating the development of biomaterial strategies to improve cell survival *in vitro* and *in vivo*. For example, Matrigel was mixed with molecules that prevent anoikis and apoptosis, successfully delivering hESC-CMs into the ventricles of infarcted rat hearts (37). Although readily available, Matrigel is not clinically relevant because it is derived from the basement membrane of a mouse sarcoma, motivating the development and use of other natural and synthetic hydrogels, such as alginate (38), polyethylene glycol (39), self-assembling peptide hydrogels (40), fibrin gels (41, 42) and collagen:chitosan blends (43).

Another important aspect of cell-based therapy is the demand for large cell quantities. Typically, 10^6 cells are transplanted in mouse MI models (44) (0.15 g heart) (15), 10^7 in rat (37, 45) (1 g), 10^8 in guinea pig (14) (3 g) and 10^9 for non-human primates (37–52 g) (15). Based on this scaling, clinical application in humans (300 g) (15) would require $10^{10–11}$ cells for sufficient engraftment of the infarct area. The expense and time required for such extensive cell expansion and differentiation are prohibitive and therefore unlikely to be undertaken for a large sample pool. Extremely low cell retention and survival are the

primary causes for the large cell requirements, and if addressed could significantly reduce the cell demand (42, 46). For significant improvements in both cell survival and retention, the solution may be a graft with tissue-level connections and a high-level of vascular organization for immediate perfusion, thus motivating studies in cardiac tissue engineering and the development of biomaterials that can promote cell survival both *in vitro* and *in vivo*.

Overall, current consensus in the field is that due to potentially unlimited cell quantities and the ability to give rise to bona fide CMs in *vitro*, hESC- and iPSC-CMs are a preferred cell source for *in vitro* modelling of cardiac physiology and disease, while adult cell sources remain highly explored in clinical studies due to demonstrated safety.

3. Tissue engineering and biomaterials for the restoration of cardiac function *in vivo*

Tissue engineering methods have the potential to provide the means of delivering appropriate cells, by co-injection with a biomaterial or as a lab-grown tissue, to the damaged heart for the purpose of restoring cardiac function lost due to injury from MI or disease (Figure 1). Ideally, both the beating CMs and the vasculature should be restored upon intervention, which should be minimally invasive. Below, we review recent bioengineering advances related to the development of supportive matrices and fully functional engineered cardiac tissues. We specifically focus on myocardial regeneration as an alternative to bioengineering methods for heart valve replacement, highlighting animal models and clinical studies. We will also focus on the vascularization of engineered myocardial tissues, a requirement for both *in vitro* and *in vivo* survival.

3.1 Acellular biomaterial implants

Application of biomaterials alone to the myocardium has been shown to reduce adverse changes in the heart geometry (cardiac remodeling) post-MI in both small and large animal models (47–49), presumably due to the ability of the biomaterials to stabilize the mechanical properties of a thinning ventricular wall. Natural biomaterials—e.g. alginate, collagen, gelatin, chitosan, decellularized extracellular matrices (ECMs) and fibrin glue—have been used to enhance the mechanical strength of the ventricular wall, with or without growth factors (VEGF, bFGF, HGF) immobilized to improve wound healing and cell survival (50–52).

Cohen's group developed a minimally invasive method of delivering alginate into the infarct site, which was demonstrated to reduce left ventricle enlargement in a swine MI model (38). In this study, biotin-labeled alginate was injected into the coronary artery through a catheter without open heart surgery. The alginate solution diffused out of the infarcted leaky vessels into the damaged myocardium where a high concentration of calcium ions, released due to cell damage, induced the alginate to undergo a liquid to gel transition (53). Because the method is simple and minimally invasive, and the material requires few manipulations *in vitro*, the treatment was approved by the FDA for clinical trials. Currently, there are two Phase II clinical trials with alginate biomaterials for treatment of acute MI (54, 55).

VentriGel, a hydrogel composed of decellularized porcine ECM, is also progressing towards clinical trials following successful pre-clinical study in pigs that demonstrated attenuated pathological remodelling and improvements in contractile function with gel injection into the heart upon MI (56).

However, acellular biomaterial treatments do not provide a long-term regenerative solution because they do not supply a means of replacing the millions of lost cells, the hallmark of cardiac regeneration. In addition, biomaterials with long degradation cycles could cause adverse remodelling due to the presence of non-contractile or poorly contractile heart wall regions.

3.2 Engineering heart tissues

Engineered heart tissue (EHT) has the potential to restore cardiac function (57) based on proof-of-concept studies that demonstrated implanted EHTs could be functionally integrated with the host heart (58, 59) and improve heart function post-MI (57, 58, 60). This was first demonstrated 10 years ago, in the landmark study by Zimmermann *et al*[43] wherein implantation of EHTs on the epicardial surface of the heart was clearly shown to result in functional improvement post-MI. Since *in vitro* approaches aim to organize cells into functional tissues, common functionality bench-marks include the ability to generate a contraction force (25 mN/mm^2 for healthy human adult tissue (61)) and propagate electrical signals (~43 cm/s for healthy human adult tissue (62)). According to a classic tissue engineering paradigm, cells and scaffolds in combination are cultivated in a bioreactor in order to achieve a desired degree of functionality. Neonatal rat CMs are commonly used as a model system (63) but the use of hESC-CMs has been gaining momentum, as the differentiation protocols are improved (64–66). Porous or fibrous scaffolds made from synthetic materials (PGA/PLLA, PGS (67, 68)) as well as natural materials (alginate (69), collagen (70), chitosan (71)) are often used (72). The use of hydrogels has also been reported (38), (73). In recent years, decellularized heart emerged as an attractive scaffold in the form of a porous material (74, 75) or hydrogel (76) for CM cultivation since the main components of the cardiac ECM and architecture were preserved during the decellularization process. Additionally, advances in microfabrication and patterning of synthetic materials have enabled the creation of tissues with a high degree of anisotropy (77–80).

Nanotechnology techniques have been adopted into cardiac tissue engineering to enable the production of scaffolds that aid in functional cardiac tissue formation. For example, gold nanowires were incorporated into porous alginate scaffolds to improve scaffold conductivity for cardiac cell culture (81). To mimic the coiled fibers of the native heart matrix as well as high electrical conductivity, neonatal rat CMs were cultivated on scaffolds generated from electrospun poly(ε-caprolactone) micro-fibers doped with gold nanoparticles (82). Tough, yet flexible scaffolds with enhanced electrical properties were created by incorporating carbon nanotubes (CNTs) into aligned poly(glycerol sebacate):gelatin electrospun nanofibers (83). To form biohybrid actuators, carbon nanotubes were incorporated into gelatin methacrylate for cardiac cell culture (84). Nanotechnology techniques can also be used to help monitor the function of engineered cardiac tissues, e.g. a mesh of silicone

nanowires was incorporated into fibrous PLGA scaffolds to enable real-time monitoring of electrical activity of cardiac constructs (85).

EHTs can also be created without a scaffold using stacked CM monolayers. The cells are first grown on poly(*N*-isopropylacrylamide)-grafted polystyrene dishes. Lowering the temperature from 37 °C to 20 °C resulted in a hydrophobic to hydrophilic transition of the surface, which released the cell monolayer. Transplantation of these cell sheets onto the epicardial surface of infarcted rat hearts (86) or failing human hearts (87) improved cardiac performance.

External biophysical stimulation can be used to enhance the maturation levels of hESC-CMs or hiPSC-CMs that are generally considered immature compared to the terminally-differentiated adult human CMs (88, 89). Long-term (up to 6 months) monolayer culture has been demonstrated to push hESC-CMs and hiPSC-CMs toward higher maturation levels as assessed by morphology and subcellular organization, including myofibril density, alignment and Z-disk registration (90). In terms of function, studies clearly demonstrated that physical stimuli, such as mechanical (73) or electrical (91–93), are important to the morphology and function of EHTs.

Hirt *et al* (94) presented similar results in a recent study demonstrating the beneficial effects of long-term electrical stimulation. Stimulated hydrogel-based EHTs exhibited an improved cardiac tissue structure and function, including higher contraction force, denser CM networks, better gap junction coupling, a physiological response to external calcium ion stimulation and an increased inotropic response to isoprenaline.

The fetal heart experiences mechanical stress very early and mechanical stimuli may trigger sarcomere development in CMs resulting in maturation and physiological hypertrophy. To simulate the *in vivo* maturation condition, Tulloch *et al* (95) carried out both static and cyclic mechanical stimulation using a FLEX Cell device, demonstrating an improved cell and matrix alignment approximating a native cardiac muscle.

Because of the interconnected nature of electrical and mechanical signals *in vivo*, it has been postulated that a combination of both electrical and mechanical stimulation could provide a better isovolumetric contraction *in vitro* by triggering the appropriate dynamics between stretch and contraction (96). Recently, Morgan and Black (97) built a bioreactor platform with integrated electrodes and mechanical stretching driven by compressed air that enabled controlled electrical and cyclic mechanical stimulation. It was observed that electrical or cyclic stretching alone had similar effects on the tissue, but the most impressive effects were observed with delayed combined stimulation (electrical pacing starting 0.49 s after mechanical stretching) and resulted in an increase in the expression of SERCA2a and cardiac troponin-T, proteins responsible for calcium handling and contractility. The results indicate the importance of combined electromechanical simulation and the selection of appropriate timing for stimulation (97). Similarly, Miklas *et al* (98) combined electrical pacing with static stretching and found improved sarcomere structure and increased contractile force.

The optimal level of maturation that EHTs should achieve before implantation is still to be determined. While a more mature cell population is clearly desired in terms of electrophysiological matching with the native adult human tissue, maintaining the viability of fully matured CMs or cardiac tissues during handling and implantation may be more challenging due to their higher metabolic demands. In contrast, a tissue composed of less mature cells may be more robust and due to its higher plasticity may aid in survival upon implantation. However, the risk of improper functional coupling after implantation and therefore the incidence of arrhythmias may be higher with EHTs composed of less mature cells.

3.3 Vascularization

One important factor for EHT implant survival is rapid vascularization and the establishment of a mature, fully functional vasculature that is integrated with the host tissue. Upon MI, the infarcted area can cover as little as several mm to as much as 50% of the left ventricle, a fatal scenario (99). In the case of reparable damage, the required EHT would preferably be of equivalent size or larger. For repair of infarcts that span the entire thickness of the ventricular wall, EHTs up to 1 cm in thickness would be required. However, engineered tissue thicker than 500 μm will suffer from insufficient transfer (mainly by diffusion) of oxygen (100) and nutrients for the high volumetric consumption rate (67). CMs require a particularly high level of oxygen supply to support their continuous beating action (67, 100, 101). Based on the average oxygen consumption rate of CMs of 26.7 nmol/min/mg protein, engineered tissues containing a high cell density (e.g. 10^8 cells/m^3) can be expected to consume oxygen at the rate as high as 10 μM/s (102). Coupled with low oxygen solubility in an aqueous solution (220 μM at normoxia and 37 °C) devoid of oxygen carriers (e.g. hemoglobin in blood), without perfusion, constant media oxygenation or a means to increase total oxygen concentration in the culture media, all oxygen would be depleted in a mere 22 s.

Therefore, one of the great challenges to engineering healthy and functional EHTs is supplying CMs in the tissue with adequate oxygen and nutrients via vascularization. The high metabolic rate of CMs is supported by a high capillary density in the native myocardium (103). In addition, the presence of a vasculature along with supporting cells such as fibroblasts, may support CM survival indirectly through paracrine signaling and gap junctional coupling (104–106). We focus here on reviewing vascularization approaches relevant to the production and implantation of EHTs and refer the reader to other excellent reviews on the general topic of cardiac revascularization (107–109).

Initial solutions to the problem of vascularization were derived from biological methods (i.e. growth factor delivery, gene therapy, cell therapy, etc.) that attempted to stimulate endogenous blood vessel growth into the infarcted myocardium but these approaches met with only limited success (110–113). For example, the delivery of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), by protein or gene therapy was found to cause the formation of a highly disorganized and leaky vasculature (114). These methods are further limited by complicated pharmacokinetics, the high cost of factor levels, the requirement for localized effects and in the case of gene therapy, low *in vivo* transfection

rates (115, 116). MicroRNAs, small non-coding RNAs, were recently successfully employed to direct myocardial angiogenesis (117), (118). However, it seems unlikely that endogenous endothelial repair alone can regenerate a major infarct or a fully-formed scar tissue (119).

In a pre-vascularization approach, cardiac patches with angiogenic factors were placed on the omentum for one week prior to inducing an MI, and then transferred to the heart post-MI (69). While improved cardiac function was demonstrated 4 weeks post-MI in the omentum-generated cardiac patch group, this approach has some disadvantages including the need for two invasive surgeries (implantation and removal from the omentum) and the temporary cessation of blood flow to the tissue during surgery.

Biomaterial-based vascularization methods have also been investigated and include porous collagen scaffolds with (50, 120, 121) and without (122) covalently-immobilized angiogenic factors, alginate with two growth factors (VEGF and PDGF-BB) sequentially released, and decellularized porcine myocardial matrix (123), all of which were demonstrated to induce angiogenesis when placed on the myocardium. Additionally, co-culture of ECs and CMs or tri-culture of ECs, FBs, and CMs are now accepted as viable methods to enhance patch survival and anastomosis, partly through the cross-talk between ECs and CMs that improve CM survival and spatial organization (124–128).

Engineering-based vascularization strategies include the fabrication of branching templates on which ECs assemble. Borenstein *et al* (129), (130) first fabricated such templates by silicon etching using polydimethylsiloxane (PDMS) molding. An *in vitro* perfusable 3D microtissue cardiac bundle model was also developed using a polytetrafluoroethylene (PTFE) tubing template (131). However, materials such as PDMS and PTFE are non-biodegradable and thus have limited usefulness *in vivo*. Consequently, the field has shifted to the use of biocompatible and biodegradable materials such as poly(lactide-co-glycolide) (PLG) and poly(glycerol sebacate) (PGS). The mechanical strength and the biodegradable properties of PGS allow it to be molded into a vascular network using standard soft lithography techniques (132). A subtractive method is commonly used to create perfusable vessels in hydrogels, wherein a sacrificial vascular network is first created from e.g. gelatin (133, 134) and then embedded within a cell-laden hydrogel. The gelatin is then removed by increasing the temperature of the construct, leaving behind open channels for endothelialization. A mechanically stable carbohydrate glass was also used to rapid cast a patterned vascular network in hydrogels (135). While promising, these hydrogel-based methods have their own shortcomings, which include the inability to reach physiological cell density within the gel while preserving the open lumens of the engineered vasculature and the inability to apply this architecture *in vivo* in a minimally invasive manner.

To address these limitations, biological and engineering methods have been combined in attempts to design microfabricated scaffolds that can guide tubulogenesis. Chiu *et al* (136) used a collagen-chitosan blend hydrogel to control the release of thymosinβ4 (Tβ4), and induced recruitment and differentiation of ECs and smooth muscle cells (**S**MCs) in epicardial capillary outgrowths *in vitro* and augmented angiogenesis *in vivo* following subcutaneous injection (136) (137). When administered through intra-myocardial injections

to infarcted rat hearts, the Tβ4-encapsulated gel was demonstrated to significantly decrease viable tissue loss and to maintain wall thickness post-MI (137). A microgroove pattern coated with Tβ4-encapsulated hydrogel could direct capillary sprouting between an explanted artery and vein and the addition of VEGF and hepatocyte growth factor (HGF) accelerated the sprouting processes and resulted in lumen formation (138). Neonatal rat CMs seeded around the engineered vasculature exhibited improved function and enhanced cross-striations. However, it remains a challenge to form fully functional vascular beds that can be injected and can integrate quickly with the host vasculature. Vascular integration is a necessity to sustain the implanted cells/tissue, which can then allow for the much slower process of muscle fiber electrical coupling between the implanted cells/tissue and the host tissue (139).

4. Applications of tissue engineering in modeling of cardiac physiology and drug discovery

Tissue engineering could also provide a new paradigm for pre-clinical drug development programs by providing physiologically validated human cardiac substrates (Figure 1). Ideally, bioengineering methods can provide arrays of functional and mature human myocardial tissues for screening the side effects of drug candidates and the discovery of new therapeutic agents. Such tissue arrays should be compatible with standard screening practices that use well plates, electrodes to evaluate electrophysiological properties and standardized imaging equipment such as microscopes or high-content imaging instruments. These tissue engineered platforms, may be most useful prior to initiation of animal and clinical studies, in the target validation and lead optimization stages of drug development.

4.1 Engineering cardiac microtissues

Thousands of human cardiac tissues may be required per day in order to satisfy the demand of the pharmaceutical industry in the drug screening process. Even with the use of hESCs or iPSCs as a source of CMs, cell production would quickly become an issue in the envisioned approach. Hence, tissue engineers focused on creating miniaturized 3D microtissues (~500 μm in width) usually by means of cell-gel compaction in a temperature-curing hydrogel. Zhao *et al* (140) developed a microtissue platform to investigate the mechanical properties of engineered tissues and found that the stiffness of the tissue is mostly contributed by the ECM structure, which is reorganized by the cells. To mimic important cues present during embryonic development such as ECM composition, tension and tri-dimensionality, Nunes *et al* (89) used microfabricated wells (Figure 3A) of constant height and width but variable length to house a surgical suture in a multiwell plate (Figure 3B) that would serve as a template to guide collagen type I gel compaction. Gel compaction (Figure 3C–D) promoted the alignment of the hESC-CMs and hiPSC-CMs in the 3D microtissues (Figure 3F), termed “Biowires”, such that they resembled cardiac myofibers. The platform also permitted electrical pacing to be provided to the cardiac Biowires through electrical field stimulation (Figure 3F) and the study of the effect of applied drugs (e.g. epinephrine) on contractile properties and impulse propagation (Figure 3F). The biomimetic cues provided by the Biowire platform promoted maturation of the CMs, as determined by an improved ultrastructural organization of the myofibrils, significantly increased conduction velocity,

increased cell size and aspect ratio, decreased proliferation rates, improved electrophysiological and Ca^{2+} handling properties, and significantly reduced number of CMs with automaticity. The small size of these cardiac Biowires allows for (i) maintenance in culture without the need for perfusion, (ii) the use of low cell number and (iii) compatibility with multiwell plates (Figure 3B), suggesting that Biowires may be used as an *in vitro* platform for drug screening.

Alternatively, Thavandiran *et al* (141) used a microfabricated platform to engineer cardiac microwires (**CMWs**) composed of 100,000 cells. The 24-well plate platform was designed such that five CMWs could be generated per well providing a total of 120 tissues per plate. Notably, this study closely investigated the cell composition of the cardiac tissue input population. Primary cells are a heterogeneous mixture of native heart cells that can self-assemble, whereas hESC-derived heart cells have undefined "structural" cell types. An understanding of the contributions of these cells to overall tissue organization and function is lacking. A computational model of intra-tissue stress and sarcomere contractility was also developed to biomechanically advise the final tissue mold design (Figure 4A), a model that could be applied in the design of *in vitro* models for other tissue types. Thavandiran *et al* were also able to identify an optimal hPSC-CM: cardiac FB ratio for the input population, as assessed by tissue morphogenesis and cardiac maturation (Figure 4B). The expression of many of the contractile markers of CMs (including MYL2, MYL7, MYH6, and MYH7) reflected improved maturation (142). Furthermore, Thavandiran *et al* (141) used their design criteria to create a disease model, a tachycardic model of arrhythmogenesis, by forming geometrically distinct circular tissue that could be defibrillated by electrical stimulation from a state of arrhythmia to a normal rhythm (Figure 4C).

Similarly, Hirt *et al* (143) reported an *in vitro* model to simulate the pathological cardiac hypertrophy induced by increased afterload by initially casting a fibrin-based EHT between two hollow elastic silicone posts. After 2 weeks, the posts were reinforced with metal braces in order to increase the afterload. This modification was sufficient to induce pathological cardiac hypertrophy, increase collagen type I deposition, reduce contractile function and impair diastolic relaxation. These 3D microtissue platforms therefore present a unique opportunity for creating a controllable microenvironment, healthy or disease-specific, in which to investigate various aspects of cardiac pathophysiology.

The requirements for an EHT used for cardiac-specific toxicity screening differ from those of an EHT used for functional repair *in vivo*. Since the EHTs used in drug toxicity studies require high-fidelity mimicking of the human adult cardiac tissue, the cells should ideally be terminally differentiated and fully mature. Drug testing also requires tissue growth automation, miniaturization and the ability to manufacture it in large quantities with reproducible standard dimensions and physiological properties.

4.2 Personalized medicine and drug discovery

Most of the current studies using iPSC-CMs for disease modelling have been performed in monolayers. Cardiac tissue engineering could potentially offer a new range of readouts and improve the maturity of iPSC-CMs. Using patient-derived iPSC-CMs, Liang *et al* (144) demonstrated that susceptibility to cardiotoxic drugs differed among healthy individuals and

patients with hereditary long QT syndrome (LQTS), familial hypertrophic cardiomyopathy (HCM) and familial dilated cardiomyopathy (DCM), indicating the utility of this method in drug development and screening. Similarly, Wang *et al* (145) used patient-derived iPSC-CMs to investigate the mitochondrial cardiomyopathy underlying Barth syndrome (Figure 5A–C); Carvajal-Vergara *et al* (146) investigated patients with LEOPARD syndrome, a major phenotype of which is hypertrophic cardiomyopathy (Figure 5D–E); Yazawa *et al* (6) investigated LQTS patients with Timothy syndrome (Figure 5F–K); Moretti *et al* (147) investigated a family with LQTS type I caused by a specific missense mutation; and Sun *et al* (148) investigated a family with an inherited form of DCM. In all cases, the patient-derived hiPSC-CMs recapitulated the major disease phenotypes, and in some cases the disease models were used to provide insight into the molecular mechanisms underlying the pathology (6, 145, 146) or as drug testing platforms (6, 148) (Figure 5).

The entire drug development process, from target discovery to FDA approval, takes an average of 15 years and costs the pharmaceutical industry an average of $1.5 billion. Despite the heavy investment, there are still drugs withdrawn from the market due to serious toxicities and adverse effects on the cardiovascular system, such as Tegaserod (Zelnorm), Sibutramine (Reductil/Meridia), Propoxyphene (Darvocet/Darvon) and Rosiglitazone (Avandia) (3). To weed out unsafe drugs more efficiently, 3D microtissues could be used to screen for the cardiotoxic effects of pharmaceutical agents as well as their liver metabolites *in vitro*. In addition to testing compounds that bind to the hERG channel, which is a major concern in cardiac safety testing due to the ability of hERG-binding compounds to cause fatal arrhythmias (149), engineered tissue substrates could give more integrated contractility readouts and delineate rhythm disturbances due to interference with other ion channels and contractile proteins as well. Importantly, multiple ion currents—sodium (I_{Na}), L- and T-type calcium (I_{CaL} and I_{CaT}, respectively), inward rectifier potassium (I_{K1}), rapid and slow activating components of the delayed rectifier potassium (I_{Kr} and I_{Ks}, respectively), transient outward potassium (I_{to}), and “funny” pacemaker (I_f)—were recorded in iPSC-CMs at physiologically relevant levels (150). There is also some evidence that the representative ion currents (e.g. hERG channel and I_{K1}) were enhanced using cardiac tissue engineering techniques (89) as compared to cultivation in embryoid body controls.

4.3 Functional readouts enabled by cardiac tissue engineering

The availability of 3D human cardiac tissues motivates the development of novel techniques for non-invasive tracking of tissue function and contractile response. Patch clamping is most accurately used to test individual ion channel conductance, even in a high-throughput manner, with single-cell suspensions (151), therefore it cannot be applied in a non-invasive manner to a 3D tissue. Recently, multi-electrode arrays (MEA) were utilized to detect the action potential (AP) changes in monolayer or 3D aggregates (152). Braam *et al* (153) showed that the field potential measured by an MEA can reveal changes in drug-treated CMs. Clements and Thomas (154) used hESC-CMs in a 48-well format MEA analysis to generate multi-parameter data in order to profile the effects of 21 different compounds targeting key cardiac ion channels. They were able to demonstrate improved risk assessment over single-parametric approaches, indicating the validity of designing multi-parameter screening platforms. However, the drawbacks include the vague resemblance of the self-

aggregated cell spheroid used to the 3D structure of the native ventricle; the complexity of obtaining an integrated AP profile in 3D; and the possibility of tissue damage resulting from inserting the MEA into the tissue after tissue formation.

Velocity of impulse propagation and calcium handling are commonly assessed by optical mapping in 3D cardiac tissues (155–157). Due to the toxicity of either the voltage- or the calcium-sensitive dye and the non-sterile environment, the assessment is a terminal stage evaluation that cannot be used for long-term observation (155). Conversely, the xCelligence RTCA Cardio Instrument is a label-free, real-time system for dynamic monitoring of CM beating and cardiotoxicity assessment. The gold microelectrode plate at the bottom of culture well is used to measure the changes of impedance of the monolayer tissue while beating. The company presented impressive data on toxicity screening of drugs with known effects (158). Unfortunately, their technology can only assess 2D monolayers and the data is an indirect measurement of tissue contractility, which cannot be translated into actual force measurements (158).

Contractile assessment of 3D cardiac tissues includes measurements of contraction force, beating frequency, as well as contraction and relaxation duration. It is hypothesized that these properties of a cardiac microtissue could be related to cardiac output (63, 73). Because of their small size, engineered cardiac microtissues are fragile and easy to damage when handling, and therefore most research groups work with optical tracking to minimize the manipulation during culture and analysis. Measuring changes in cell or tissue area during contraction using image analysis does not provide an absolute value for the force generated (91). However, optical tracking could be used to measure the deflection of posts around which cardiac tissue is grown to obtain an absolute value for the contraction force (141, 159).

Muscular thin films (MTF) are composed of a 2D layer of engineered muscle grown on one side of a PDMS film. The thin film undergoes deformation as a result of contraction when electrically stimulated. Optical recording of MTF contraction is filmed and the contractile forces of the tissue are calculated by analyzing the recording using a mathematical model (160, 161). This platform can be used for both smooth and striated muscle models. However, because the MTFs consist of an oriented monolayer of cardiac cells, they are more representative of 2D cell layers rather than 3D solid tissues.

Eschenhangen *et al* (162) developed a drug screening platform in a 24-well format, in which hPSC-CMs were seeded with fibrin enhanced ECM. Each well included two silicone posts for tissue formation and functional testing. Beating of the tissues resulted in the inward deflection of the posts and the degree of deformation could be correlated to the contractile forces using mathematical models, as the stiffness of the PDMS can be determined by standard mechanical testing protocols. The system was able to realize long-term monitoring of tissue contractile force, contraction and relaxation duration, and beating frequency in a sterile manner (163–165). In terms of drug screening, digoxin toxicity was confirmed at both 100 nM and 1000 nM using force measurements; and repolarization inhibitors, such as chromanol 293B, quinidine and erythromycin, were found to induce significantly prolonged diastolic times at 1000 nM.

Boudou *et al* (159) also used a PDMS post system to study the relationship between EHT quality and the stiffness of the posts and collagen matrix. They miniaturized the posts down to 1 mm in length and were able to produce 200 EHTs with 1 million CMs. Dose response to both isoproterenol and digoxin was tested in this platform. Both ionotropic and toxic effects of digoxin, consistent with the literature, could be demonstrated in this platform (166). Similarly, the effects of isoproterenol were consistent with the literature showing increased contractile forces at a low dosage and decreased forces at a high dosage.

Another study introduced fluorescent microbeads (167, 168) on top of the posts and reduced the scale significantly to the micrometer level to facilitate the image processing strategy. Magnetic beads have also been incorporated into posts to facilitate magnetic stretching and thereby, accelerate tissue maturation (140). Rodriguez *et al* (169) recently designed a PDMS-based micropost array platform for the assessment of CM contractility using optical tracking of the post locations that enabled measurement of twitch force, velocity and power.

The limitation of these contraction-based platforms is that the setup can only measure contractile forces along one dimension. Also PDMS is much stiffer (~1 MPa) than the native myocardium (~10–100 kPa), which may trigger pathological hypotrophy in the EHTs. Another commonly recognized limitation is the high hydrophobicity of PDMS, which is linked to its ability to absorb small hydrophobic molecules motivating the development of novel materials, e.g. polyurethanes to replace PDMS (170).

5. Conclusions and Future Challenges

Cardiac regenerative medicine has made significant progress in recent years. Successful pre-clinical studies have demonstrated the integration of cardiac patches to recipient hearts and the ability to improve contractile function. A cellular biomaterials, such as alginate and decellularized ECM, are already in clinical studies and challenges related to cell retention are being addressed using novel biomaterials. In terms of vascularization, EHTs with pre-established, perfusable, 3D, continuous vasculature can be potentially scaled up to clinically relevant size and thickness. In addition, immediate integration into the host circulation by *in situ* anastomosis may be needed to significantly improve the survival of CMs in implants.

Cardiac tissue engineers are also developing human cardiac microtissue platforms that can offer non-invasive readouts of contractile force and use small cell numbers per tissues (e.g. 100,000–500,000 cells). Coupled with iPSC technology, these platforms can offer improved disease modelling tools. Current drug toxicity screening requires microtissues that closely mimic human adult cardiac tissue. To achieve a high level of maturation, a fully established protocol with biochemical, electrical and mechanical stimuli should be designed to push tissue towards a fully mature stage. In addition, due to the fact that the vasculature contributes a great degree to drug distribution *in vivo*, it would be physiologically relevant to have a miniaturized integrated cardiovascular system *in vitro* to facilitate toxicity screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Tissue engineering as it relates to cardiac regenerative medicine has made exciting progress in recent years, which has opened up the possibility of more wide-spread applications than previously imagined. It now seems plausible that in the near future bioengineering methods could be used to not only regenerate damaged myocardium but also facilitate drug screening and discovery, as well as to create personalized medicine testing platforms. The state of the art, challenges and perspectives are discussed.

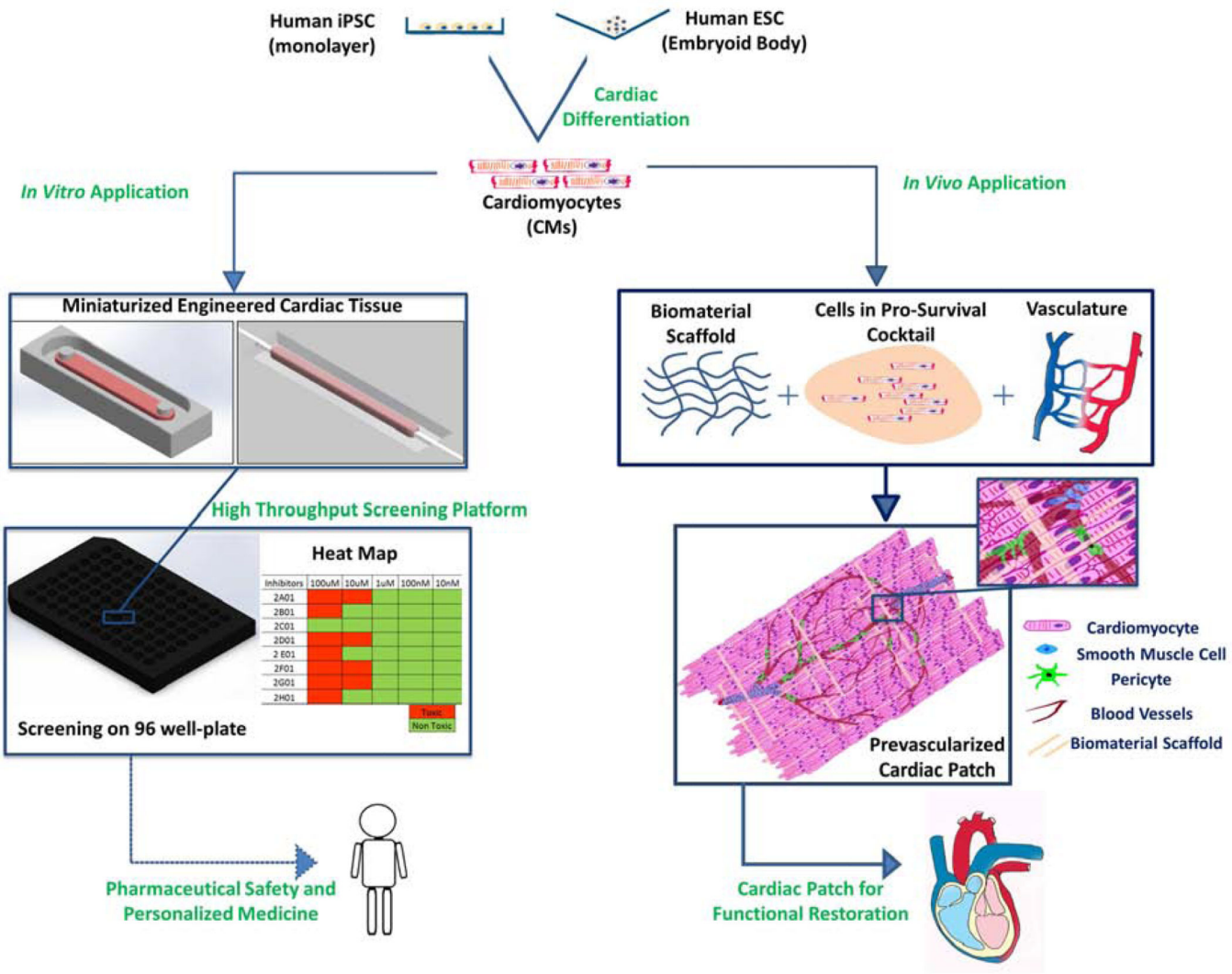

Figure 1. Overview

Human induced pluripotent stem cells (iPSCs) and human embryonic stem cells (ESCs) are capable of differentiation to produce cardiomyocytes (CMs), which can be applied to both *in vitro* and *in vivo* applications. *In vitro*, miniaturized cardiac tissues are engineered in large numbers using small amounts of cells and reagents. These microtissues are used in platforms such as customized 96-well plates with topographical cues, e.g. wires or posts that guide tissue assembly and enable read-out of contractile force. The data are analyzed to evaluate efficacy and safety as part of the pharmaceutical development process. The same strategy can also be used to optimize therapeutics for personalized medicine. *In vivo*, CMs are combined with a pro-survival cocktail, biomaterial scaffold and pre-established vasculature to generate functional cardiac patches that allow for immediate perfusion and electromechanical coupling between the patch and the host tissue after transplantation for true cardiac functional restoration.

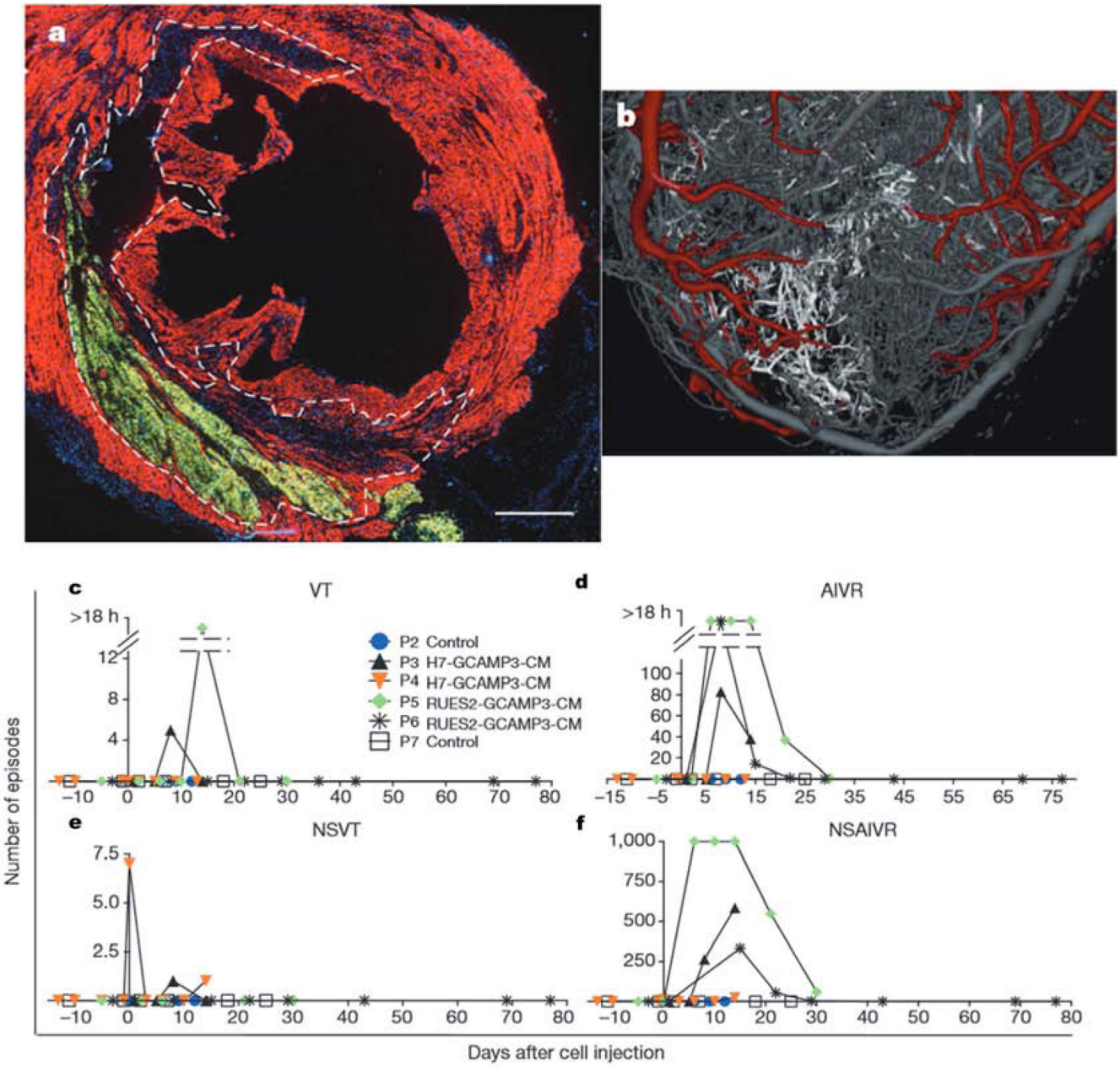

Figure 2. ***In vivo*** **study of hESC-CM therapy in a non-human primate model**

(A) Remuscularization of the infarcted macaque heart with hESC-CMs 14 days after injection. Dashed line represents the infarct area, including hESC-CMs expressing GFP signal (green) and contractile protein α-actinin (red for both human and monkey). Scale bar: 2000 mm. **(B)** 3D tomography of a heart perfused with Microfil at 3 months post transplantation. Red indicates perfused vessels in the infarct site; grey indicates vessels in the host tissue and white indicates new vessels within the graft. **(C)** Ventricular tachycardia (VT). **(D)** Accelerated idioventricular rhythm (AIVR). **(E)** Non-sustained ventricular tachycardia (NSVT). **(F)** Non-sustained accelerated idioventricular rhythm (NSAIVR). P2–7 denote sample names. Control animals (P2 and P7) had no episodes of arrhythmia whereas

all samples with transplanted hESC-CMs had either long duration (18 h per day) VT or AIVR or multiple episodes of arrhythmia within the first 30 days post-injection. (15)

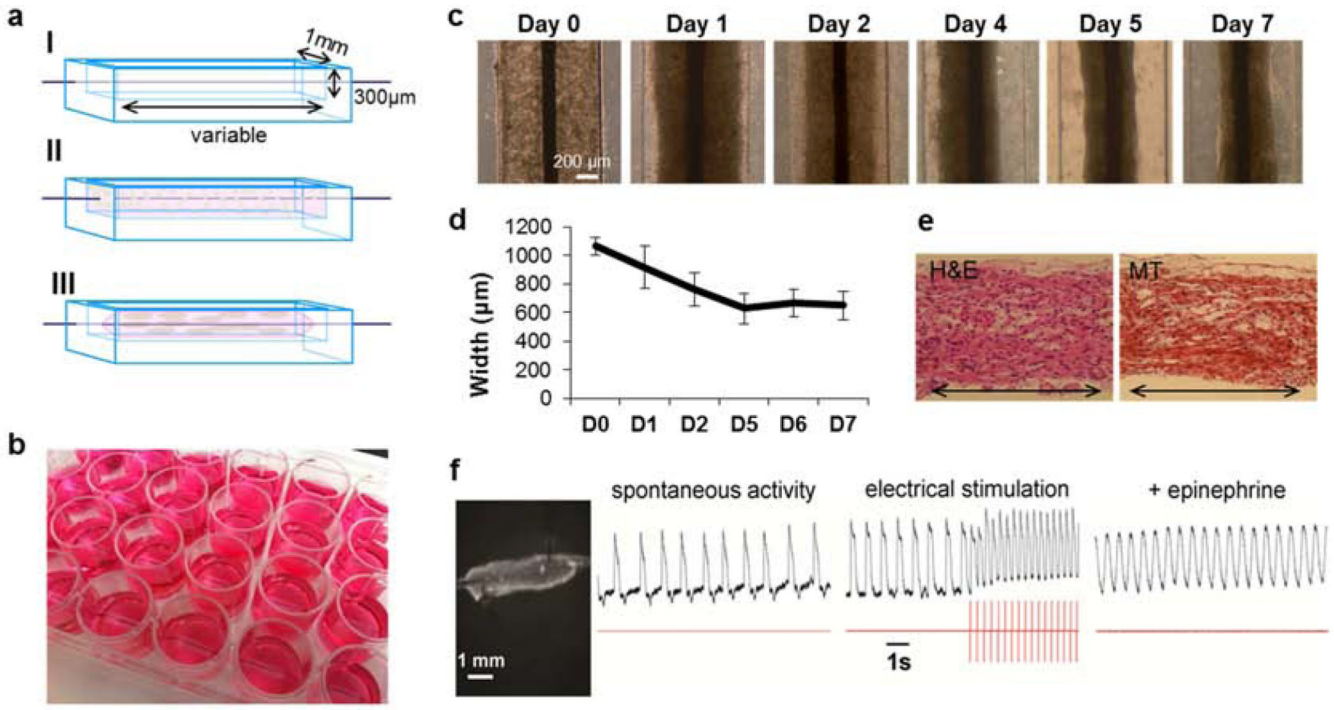

Figure 3. Biowire microtissues

(A) Biowire assembly platform. *I*. Surgical suture (black) is placed in the center of the PDMS channel. *II*. Cardiomyocyte and collagen type I gel suspension is seeded into the main channel around the suture. *III*. Pre-culture of hESC-CMs in the template allows the cells to remodel the collagen and contract around the suture to generate human cardiac "Biowires". **(B)** Biowire PDMS platforms assembled in a multiwell culture plate. **(C)** Brightfield images of hESC-CMs in the Biowire template during pre-culture. **(D)** Quantification of the compaction of the gel over the pre-culture period. **(E)** Representative images of hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining for Biowire sections. Orientation of the suture denoted by the arrows. **(F)** *Left*. Representative images using a potentiometric fluorophore (DI-4-ANEPPS) of a Biowire indicating spontaneous electrical activity. Representative traces of impulse propagation recording for a Biowire without electrical stimulation (*Left* trace), with electrical stimulation (*Middle*) and in response to epinephrine (*Right*). The stimulation frequency is depicted in red. (89)

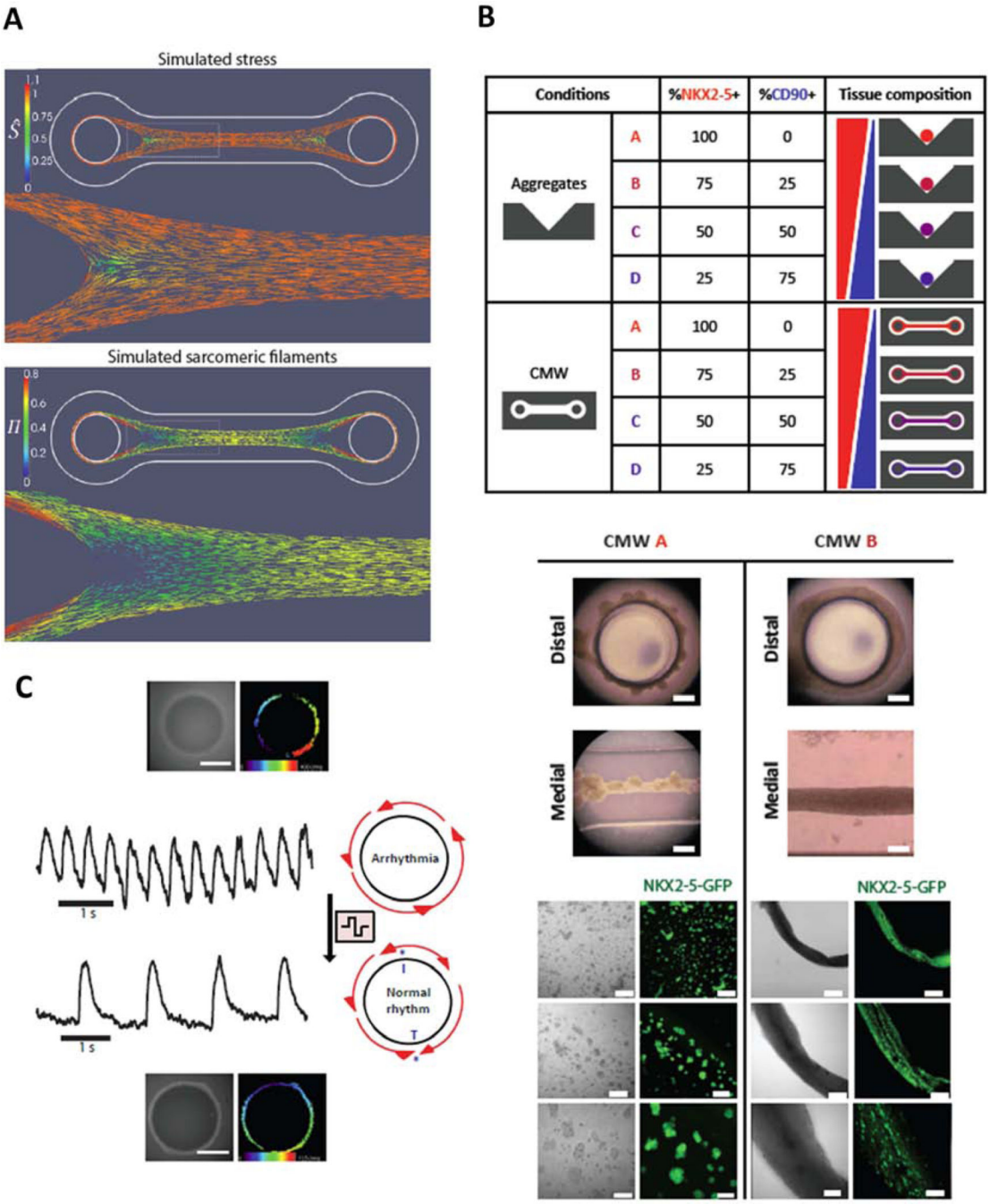

Figure 4. Cardiac Microwires (CMWs)

(A) A simulation predicting stress (represented by non-dimensional effective stress; $\hat{S}$) and sarcomeric α-actinin expression in CMWs shows a uniaxial orientation along the longitudinal axis. **(B)** *Top*, Input population for CMWs and aggregates was controlled by combining sorted NKX2.5-GFP+ (CMs) and CD90+ (FBs) cells in the described ratios. *Middle*, CMW A composed of 100% CMs formed non-integrating colonies of cells (left), whereas CMW B composed of 75% CMs and 25% FBs formed well-integrated and organized tissue. *Bottom*, bright field and fluorescent images of the CMW A and CMW B

tissues. **(C–D)** One cycle and signal tracing of a circular CMW generated to mimic a re-entrant arrhythmic wave. The impulse starting location is blue. **(C)** The non-stimulated circular CMW shows looping cycles of activation propagation. **(D)** Electrical field stimulation of 10 V induced a normal rhythm in the circular CMW. (141)

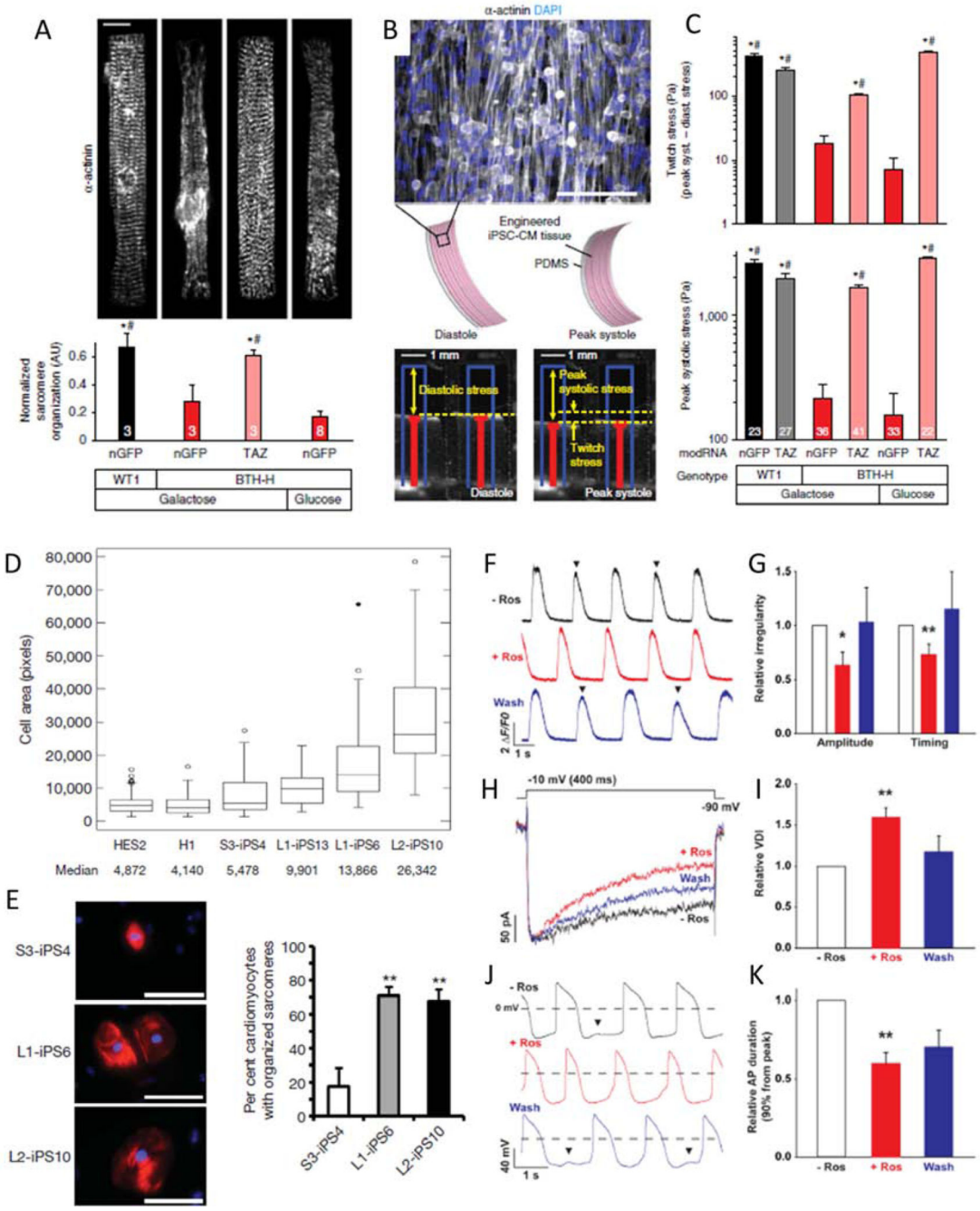

Figure 5. Patient-derived iPSCs

(A) Sarcomeric organization is impaired in patient-derived Barth syndrome iPSC-CMs. *Top*, representative images of iPSC-CMs seeded on micropatterned fibronectin, cultured in indicated medium, transfected with nuclear-localized GFP or tafazzin (*TAZ)* modified mRNA (modRNA)—a mutation in which causes mitochondrial functional abnormalities and cardiolipin deficiency resulting in Barth syndrome—and stained with α-actinin. Sarcomeres in control iPSCCMs (WT1) are regularly organized along the entire length of the cell. Sarcomeres in patientderived Barth syndrome (BTH-H) iPSC-CMs are intermittent and

sparse. Transfection with *TAZ* restored sarcomeric organization. Glucose culture of BTH-H iPSC-CMs did not rescue sarcomere formation. *Bottom*, plots quantitating sarcomere organization. Scale bars, 10 μm. Data presented as mean ± SEM. $P < 0.05$ versus BTH-H in galactose culture (*) or BTH-H + nGFP in glucose culture (#). (145)

(B–C) Barth syndrome myocardial tissue constructs recapitulate Barth syndrome myopathy. Human iPSC-CMs were seeded onto thin micropatterned elastomers supported by glass coverslips. After 5 days, the muscular thin films (MTF) were peeled off the glass and allowed to contract and curl away from the coverslip. **(B)** *Top*, α-actinin–stained image of a control iPSC-CM tissue demonstrating sarcomere alignment and fibrous structures. *Middle*, iPSC-CMs selforganize into anisotropic myocardial tissues at diastole and peak systole demonstrating a reduction in the radius of curvature of the MTF as it contracts. Scale bar, 100 μm. *Bottom*, images of MTFs in diastole and systole wherein red lines indicate automated tracking and blue lines indicate MTF length before peeling from glass. **(C)** Twitch stress and peak systolic (syst) stress of MTFs paced at 2 Hz. MTFs were generated from BTH-H and control (WT1) iPSC-CMs transfected with the indicated modRNA and cultured in galactose or glucose medium. BTH-H iPSC-CM tissues had significantly lower twitch and peak systolic stress compared to controls, a phenotype that could be rescued by *TAZ* transfection. (145)

(D–E) LEOPARD patient-derived iPSC-CMs have increased cell size and increased sarcomeric assembly indicative of cardiac hypertrophy. Stem cells (hESC cell lines: Hes2 and H1; wild-type hiPSCs: S3-iPSC4; LEOPARD syndrome hiPCs: L1-iPSC13, L1-iPSC6 and L2-iPSC10) were differentiated into CMs. **(D)** The cell surface area of 50 randomly selected troponin-T-positive cells (CMs) were measured. Boxes span 1^{st} to 3^{rd} percentile; whiskers 1.5-times median; open circles ≥1.5-times median; filled circle ≥3-times median. **(E)** Sarcomeric organization was measured in 50 troponin-T-positive CMs (red). Data presented as mean ± SD. (146)

(F–K) Roscovitine rescues the cellular phenotype of Timothy Syndrome (TS) patientderived iPSC-CMs. Roscovitine (Ros), a cycline-dependent kinase inhibitor that increases voltage-dependent inactivation (VDI) of CaV1.2, the main L-type channel in the mammalian heart was investigated as a rescue for the TS phenotype. Black: without treatment; Red: with Ros treatment; and Blue: after wash out. **(F)** Ros treatment reduced the frequency of irregular Ca^{2+} transients in TS iPSC-CMs. Arrowheads denote irregular Ca^{2+} peaks. **(G)** Ros treatment significantly reduced the irregular amplitude and timing of spontaneous Ca^{2+} transients in TS iPSC-CMs. **(H)** Inactive Ba^{2+}currents in TS iPSC-CMs were restored by Ros treatment. **(I)** Ros treatment significantly increased CaV1.2 VDI in TS iPSC-CMs. **(J)** Ros treatment decreased the frequency of abnormal depolarizing events. Arrowheads denote delays after depolarization. **(K)** Ros prevented AP prolongation in TS iPSC-CMs. Data presented as mean ± SEM. *P<0.05, **P<0.01. (6)