Abstract: Twenty years after the initial description of a tissue engineered construct, 3-dimensional human cardiac tissues of different kinds are now generated routinely in many laboratories. Advances in stem cell biology and engineering allow for the generation of constructs that come close to recapitulating the complex structure of heart muscle and might, therefore, be amenable to industrial (eg, drug screening) and clinical (eg, cardiac repair) applications. Whether the more physiological structure of 3-dimensional constructs provides a relevant advantage over standard 2-dimensional cell culture has yet to be shown in head-to-head-comparisons. The present article gives an overview on current strategies of cardiac tissue engineering with a focus on different hydrogel methods and discusses perspectives and challenges for necessary steps toward the real-life application of cardiac tissue engineering for disease modeling, drug development, and cardiac repair. (Circ Res. 2017;120:1487-1500. DOI: 10.1161/CIRCRESAHA.117.310738.)

Key Words: cardiac repair □ drug testing □ myocytes, cardiac □ stem cells □ tissue engineering

The origin of the term tissue engineering is not clear, but a 1985 proposal of Y.C. Fung to the National Science Foundation of the United States for a Center for the Engineering of Living Tissues and 2 conferences organized by the National Science Foundation in 1987 and 1988 are generally accepted as the origin of this lively and rapidly growing research field (The Emergence of Tissue Engineering as a Research Field; https://www.nsf.gov/pubs/2004/nsf0450/emergence.htm). In its 1988 definition, tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure–function relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions. Besides bone and cartilage, cardiovascular tissue engineering is the most proliferative discipline in the field (>9200 PubMed entries) and can be traced back to 1986, when the first report on tissue-engineered vasculature was published. The first engineered heart tissue (EHT) was generated 1994 by seeding chicken embryonic heart cells in collagen I between 2 Velcro-coated glass tubes to form coherently beating and force-generating biconcaval lattices. Today, 20 years later, the field is in a maturating state with EHT patches moving toward first-in-man applications, and EHT assays being on the cusp of industrial application. In the present article, we will concentrate on 3 major questions: (1) How closely do EHTs currently resemble native myocardium and how can this be improved? (2) Pros and cons of using tissue engineered heart muscle assays in drug development and disease modeling. (3) Tissue engineered heart muscle patches for cardiac regeneration. For more information also on 3-dimensional (3D) printing and organ-on-the-chip approaches, the reader is referred to recent reviews.

Technologies to Engineer Heart Muscles In Vitro

In a normal organism, parenchymal cells including cardiomyocytes are never alone. Instead, they are organized in a complex 3-dimensional tissue that, in the case of the heart muscle, is comprised mechanically and electrically connected cardiomyocytes intimately coupled to capillary endothelial cells, fibroblasts, vascular smooth muscle cells, and macrophages. A mammalian heart is composed of ≈20% to 30% cardiomyocytes and 70% to 80% nonmyocytes. The principal promise of the field is that engineered 3D heart muscles recapitulate normal tissue organization in vitro and allow the study of cell–cell interactions and heart muscle function under normal and pathological conditions. Two principally different strategies are pursued to generate tissues—(1) to capitalize on and promote the natural ability of cells to assemble and form organized 3D structures or to use the natural organ structure as blueprint and (2) to engineer the 3D organization by technical or chemical means. This review will focus on the first strategy because it is the field the authors have actively contributed. A schematic overview of different strategies is given in Figure 1. The latter is attractive particularly for engineers and offers the advantage that the desired geometric form of the final construct can be technically designed. Although seeding of cells in preformed matrices had been an issue in early work, more recent work showed good tissue formation with novel materials such as porous collagen sponges or poly(glycerol sebacate) scaffolds. Organ-on-the-chip (the heart on a chip), 3D-printing approaches, and intelligent multifunctional synthetic polymers are currently developed to advance the field in terms of miniaturization, high-throughput, simulation of organ-like cell–cell
interactions and improvement of tissue structure and function. Although most technologies are not advanced enough yet (eg, in spatial resolution) to faithfully reproduce the sophisticated multicellular structures of a normal heart muscle, the field is rapidly proceeding and offers great potential. Recent examples include the generation of a perfusable, 3D microchannel network coated with endothelial cells and supporting the growth of cardiac tissue (AngioChip), the use of a multiphoton excitation 3D printing for cardiac tissue engineering, and the generation of cardiac patches that contain microelectronic elements. For more information on 3D printing and organ-on-the-chip approaches, the reader is referred to recent reviews.

Recellularization of Decellularized Organs

An attractive idea to generate heart tissue that has gained significant public attention (eg, long article in the weekly German magazine Der Spiegel in November 2016) is to use nature’s blueprint directly by decellularizing cadaveric animal hearts and repopulate the remaining extracellular matrix with cardiac cells. Protocols for decellularization, for example, the removal of all potentially immunogenic cellular materials, are well advanced, but introducing cells back into the matrix at sufficient efficiency remains a major hurdle. Initial attempts to deliver cells by coronary perfusion failed because cells did not pass the decellularized vessel wall. In a recent study, 500 million human induced pluripotent stem cell (hiPSC)–derived cardiomyocytes were injected in a volume of the free left ventricular wall of a decellularized human heart. Cells survived in the matrix and formed an immature cardiac tissue with ≈50% cell coverage that developed a force of 250 µN. Considering a normal cardiomyocyte density of 10 to 28 million/cm³, the input cell density of 100 million/cm³ was high but yielded only a relatively small area of contracting tissue that generated a pressure of <3 mm Hg. The approach is not suited for drug testing or disease modeling but may have a long-term perspective for regeneration.

Cell Sheet Technology

3D cardiac tissues can also be generated by layering cellular monolayers on top of each other. Although monolayers can spontaneously detach from PDMS (polydimethylsiloxane) surfaces and form strip-shaped 3D tissues attached to pins, the technique has been optimized by the introduction of a thermosensitive surface material losing its adhesive properties at room temperature. Advantages of the cell sheet technology include the lack of extracellular materials and its simplicity, which is of particular importance for cardiac repair applications. On the contrary, it is not well suited for drug screening or disease modeling because monolayers would have to be attached to force transducers for functional testing. Cardiac tissue structure is good but limited to a 3-cell sheet thickness. Advancements of the methods include the integration of artificial or natural blood vessels to generate thicker tissues in vitro. The method has been successfully used to build thick tissues in vivo by multistep operations (see the section on Cell-Based Tissue Engineering Strategies).

Hydrogel Method

The hydrogel method yielded the first EHT and is currently the most used technology. It is simple and multiplexable and leads to good cardiac tissue development (Figure 2). It requires cells, a hydrogel, a casting mold, and a mechanic support. The initially liquid hydrogel is mixed with cells, forms a gel, and traps the cells in a 3D form that is provided by a casting mold. A mechanic support inserted in the casting mold is essential to provide the growing tissue with mechanical load, recognized as one of the critical factors driving cardiac tissue development and maturation (see the section on Factors Influencing Structure and Function).

**Figure 1.** Plane engineered heart tissue (EHT) on Velcro-covered rods (Eschenhagen et al², A), ring EHTs (Zimmermann et al²⁴, B), fibrin-based mini-EHT on PDMS (polydimethylsiloxane) racks (Hansen et al²⁵, C), cardiac micro tissues (CMT) on fluorescent pillars (Boudou et al³⁴, D), cardiobundles on PDMS frame (Jackman et al³⁴, Copyright © 2016, Elsevier; E), micro heart muscle (Huebsch et al⁸¹, Copyright © 2016, The Authors; F), cardiac biowires (Nunes et al¹³¹, Copyright © 2013, Nature Publishing Group; G), cardiac patch (Bian et al¹³³, H). Please note that scale bars are only representative, and sketches might not match the exact dimensions. Graphics in A, B, and D were modified from Eschenhagen et al¹³⁴ with permission of the publisher. Copyright © 2012, the American Physiological Society.
Hydrogels

The most commonly used hydrogels are the natural extracellular matrix protein collagen, mixtures of collagen and Matrigel (extracellular matrix from Engelbrecht–Holm–Swarm tumors in mice containing mainly collagen IV and laminin), and the blood-clotting material fibrin. Fibrin polymerizes faster than collagen and therefore prevents cells from concentrating on the bottom during gelatinization. Until now, no synthetic material has been identified that supports cardiac tissue development similar or better than natural hydrogels. Polyethylene glycol, hyaluronic acid, or mixtures of hyaluronic acid with alginate support cardiac tissue development but less well than collagen. Systematic variation of extracellular matrix supplements on the basis of a polyethylene glycol hydrogel revealed that collagen I, fibronectin, and laminin had the best effect on the differentiation and growth of mouse induced pluripotent stem cell–derived cardiomyocytes.

Although the principles of providing mechanical support to the growing tissue remained the same in the hydrogel techniques over time, important improvements have been introduced. Earlier systems provide a static support that allows only limited or no deflection (eg, Velcro-coated glass tubes, circular casting mold, Flexcell system, and Velcro frame). This leads to an essentially isometric form of EHT contraction, allowing the EHT to contract auxotonically and perform contractile work, the physiological form of cardiac contraction.

Rodent Versus Human EHTs

3D tissues from neonatal rat heart cells exhibit excellent cardiac tissue structure (Figures 2 and 3). With the best methods, cardiomyocytes form a dense, longitudinally aligned, highly interconnected, cross-striated heart muscle that comes close to native heart muscle (Figure 2). Electron microscopic analyses of paced rat EHTs show well-developed sarcomeres with clear A- and M-bands (Figure 3). Yet, compared with native rat heart, the regular alternating pattern of sarcomeres and mitochondria was less pronounced and mitochondria showed vacuoles. EHTs made from a native heart cell mix developed 2- to 3-fold higher active forces than those made from purified cardiomyocytes. In these experiments with neonatal rat heart cells, the main difference was a higher fraction of prolyl-hydroxylase–positive fibroblasts in the unpurified cell mix used for the generation of EHTs (≈50% versus 35%), whereas smooth muscle cells and endothelial cells (EC) were low in both. Mature EHTs made from unpurified cells contain a high density of fibroblasts, smooth muscle α-actin–positive SMC, and ED2-positive macrophages and ECM-forming capillary structures. The spontaneous formation of extensive, lumen-forming vascular structures inside 3D cardiac constructs has been recently substantiated in EHTs made from neonatal mice in which EC were genetically labeled (Cdh5-CreERT2×Rosa26-LacZ).
The data in rats and mice are principally supported by experiences with human pluripotent stem cell (hPSC)–derived cells. Thus, addition of EC or stromal cells to hPSC cardiomyocytes improved cardiac tissue formation and the formation of vascular networks.39–41 Others reported that a 40% stromal cell fraction is required for the successful generation of human EHT.42 It was, therefore, initially surprising that even apparently pure hiPSC cardiomyocytes, either from proprietary (>95% purity) or commercial sources (the latter generated by genetic selection), readily formed well-structured fibrin-EHTs with good force development.43 However, others also showed that although the purity of hPSC cardiomyocytes negatively correlated with relative force (=force per input cardiomyocyte), it positively correlated with conduction velocity, and absolute force development was essentially independent of input cell purity between 45% and 90%.44 It is possible that the small fraction of (poorly defined) nonmyocytes in hPSC cell preparations suffices to provide a stromal cell environment for cardiomyocytes to form a tissue. Alternatively, fibrin provides a more flexible environment than collagen I used by the studies above. More systematic comparisons are needed to answer the question of the optimal cell composition in human cardiac tissue engineering. Compared with EHTs from native rat heart cells, hiPSC cardiomyocytes generate less organized tissues. The ultrastructure of hPSC-derived tissues seems better developed than in standard 2-dimensional (2D)-cultured hPSC cardiomyocytes, but clearly inferior to rat EHTs or native hearts (Figure 3). In terms of function, thin rat EHTs can reach almost physiological relative forces (60 mN/mm²35) and almost normal conduction velocity (>50 cm/s35). In accordance with the less developed structure, maximal published forces and conduction velocities are still lower in hPSC-derived EHTs (≈20 mN/mm² and 20–25 cm/s35,46).

Factors Influencing Structure and Function
Factors identified early as important for cardiac tissue and force development are mechanical strain/load,24,47,48 chronic electric pacing,9,33,36,49,50 and supplementation of medium with l-thyroxin (T337,51) or, as replacement of serum, T3 and hydrocortisone.31 Chronic pacing was not only associated with larger forces but also better cardiac tissue structure58 and a positive force–frequency relationship as a parameter of improved tissue maturation.33 Maturation is one of the most important issues for the whole field. A comprehensive review on this topic is beyond the possibilities of this review, and the reader is, therefore, referred to recent reviews on this topic.52,53 Another lesson learned during the past 2 decades is that tissues get better the thinner they are. This is best seen when comparing contractile force per cross-sectional area. Literature values for rat EHTs range between <1 and 60 mN/mm²20,35 and for human EHTs between <0.1 and >20 mN/mm².35 Thus, relative forces can vary by a factor of 200. Absolute values, in contrast, range only between 0.120 and 2.3 mN for rat19 and 0.0820 to 1.5 mN for human,55 that is, a factor of 20. In other words, a major factor determining the calculated relative force of EHTs is the diameter that inversely correlates with tissue density. Optimal, near-to-physiological values were reached at a final construct diameter of 100 to 200 µm.20,35 At this size, EHTs show an almost complete coverage of the cross-sectional area by cells, whereas larger EHTs contain central parts (almost) completely devoid of cells (eg,55). Approximately 100 µm is well known as the maximal thickness of compact muscles strands inside larger EHTs24 and of constructs generated by stapling cell sheets.9 It also corresponds to work with ultrathin rat heart trabeculae (≈ 100 µm thickness), which show active forces of ≤90 mN/mm² and, in contrast to thicker

**Figure 3.** Ultrastructure of cardiomyocytes derived from pluripotent stem cells and cultured in different 3D formats (D–G) or 100 days in 2D low-density culture (C). Native rat heart (A) and rat engineered heart tissue paced for 14 days (B) are shown for comparison. Note different levels of sarcomeric organization and mitochondrial density, size, and structure. Reproduced with permission from Goldstein et al137 (Copyright © 1989, The American Physiological Society; A), Hirt et al36 (Copyright © 2014, Elsevier; B), Lundy et al135 (Copyright © 2013, Mary Ann Liebert, Inc; C), Nunes et al131 (Copyright © 2013, Nature Publishing Group; D), Kerscher et al132 (Copyright © 2016, Elsevier; E), Ruan et al76 (Copyright © 2016, American Heart Association; F), and Mannhardt et al19 (Copyright © 2016, The Authors; G) with permission of the publishers.
muscles, a positive force–frequency relationship. The data suggest that, in the absence of vascularization that normally provides a maximal capillary-to-capillary distance of 20 μm, compact heart muscle bundles cannot exceed a thickness of ≈ 100 μm. Although this conclusion provides a clear argument for the use of small constructs for in vitro application, it creates an obvious problem for upscaling of cardiac patches toward first-in-human applications (see the section on Cell Survival and Proliferation).

It is, therefore, interesting that a recent publication presented a technique that allowed the generation of (almost) complete compact muscle tissue that exceeded the aforementioned thickness of 100 μm. Continuous rocking (dynamic culture) increased the thickness of cross-sectional area to ≈ 200 μm (Figure 21). This was associated with indicators of advanced cardiac tissue maturation including a high conduction velocity (52 cm/s in rat) and activation of the mTOR (mechanistic target of rapamycin) pathway. The data argue for the critical importance of better nutrient supply to underlie the improved structure and function of EHTs under dynamic culture. Better oxygen supply could also play a role but has not been directly addressed in this study. The lack of regulation of transcript markers of hypoxia argued against the role of hypoxia in rat EHTs as did the observation that continuous electric pacing, which would be expected to increase rather than decrease core hypoxia, increased the relative cell coverage of central aspects of rat and human EHTs. On the contrary, direct oxygen supply could also play a role but has not been directly addressed in this study.

Engineered Heart Muscle Assays in Drug Development and Disease Modeling

One of the 2 goals of cardiac tissue engineering is to provide better in vitro culture models of the heart than 2D monolayers— to screen for drug effects in preclinical drug development and to model cardiac diseases, particularly in combination with patient-derived hiPSC. For these purposes, it may be less relevant whether a cardiac tissue perfectly mimics native heart tissue than whether the assay is simple, robust, and better recapitulates known functions of the human heart in health and disease than cells cultured in 2D. We will, therefore, shortly summarize the state of the art in drug screening with hiPSC-derived cardiomyocytes in 2D systems and then what has been done with EHTs and comparable 3D systems.

Potential of hiPSC-Derived Cardiomyocytes for Drug Screening

The event of hiPSC cardiomyocytes could advance preclinical drug development in 2 areas—prediction of proarrhythmic side effects of drugs and the development of (positive and negative) inotropic drugs for therapeutic applications. Both are currently hampered by the limited predictive values of current test batteries and high costs. hiPSC cardiomyocytes have several theoretical advantages: (1) They overcome the significant differences between rodent and human heart biology, for example, in terms of electric repolarization (no role of the rapid delayed rectifier Ikr in rodents, strong in human) or myofilament composition (dominance of the fast β-myosin heavy chain isoform in rodents, dominance of slow α-myosin heavy chain in humans). (2) Compared with classical hERG (human Ether-a-go-go Related Gene) assays used for the prediction of Ikr effects of drugs (recombinant cells expressing large amounts of Ikr-carrying ion channels), tests in hiPSC cardiomyocytes integrate the entirety of cardiac ion channels. (3) Tests in hiPSC can integrate population- and disease-based variability in drug responses. (4) hiPSC cardiomyocytes are immature, featuring characteristics of fetal (week 16) cardiomyocytes. Compared with adult human cardiomyocytes, they are smaller with less developed ultrastructure, lower sarcomere and mitochondria density, lower resting membrane potential, lower upstroke velocity, and smaller/absent α-adrenergic inotropic responses. Spontaneous diastolic depolarization and contractile activity add to the peculiarity. (2) Although most hiPSC cardiomyocytes acquire a predominantly ventricular phenotype, heterogeneity exists with relevant fractions of cells exhibiting atrial- or nodal-like features. Experiments in cultured cells are likely prone to more confounding factors that are more difficult to control than animal experiments. This includes cellular heterogeneity, variable culture conditions including batch-to-batch variations of medium supplements, influences of long-term culture including clonal selection, and, particularly in hiPSC, the risk of karyotypic abnormalities. The latter is an increasingly recognized problem with systematic studies detecting abnormalities of various origins in 41 out to 120 ethnically diverse human embryonic stem cell (hESC) lines. Finally, systemic effects of drugs including that of hepatic metabolites and tissue/cell accumulation over time cannot be evaluated in vitro. Nevertheless, several companies providing hiPSC cardiomyocytes entered the market (examples Cellular Dynamics International [Fuiji], Axiogenesis, and Cellartis [Clontech]), drug companies started testing these cells, and much work has been devoted to improving the maturity and thereby validity of drug tests in hiPSC cardiomyocytes.

Means to Improve the Maturity of hiPSC Cardiomyocytes in 2D or Spheric Microtissues

Several factors identified as important for EHT development (see the section on Factors Influencing Structure and Function) also improve the maturity of hiPSC cardiomyocytes cultured in 2D. They include long-term culture; variations of extracellular matrices such as Matrigel; humoral factors such as T3, IGF-1 (insulin-like growth factor-1), and corticosteroids; and mechanical and electric stimulation. It is important to note that the best published sarcomeric ultrastructure in hiPSC...
Cardiomyocytes was actually seen in cells cultured for 100 days at low density.30 Although these cells are still not normal as exemplified by a low density of small mitochondria, their sarcomeric Z-M band organization is striking. Similarly, the conduction velocity in advanced 2D culture systems was similar or better than in the best 3D (35 cm/s). Of note, these rod-shaped cardiomyocytes seem to represent only a small minority of cells in 2D cell culture (see Online Figure I in a previous study49) and might create a bias compared with 3D constructs that integrate a much greater number of cells. Still, the question arises whether drug testing in simpler 2D systems does not suffice.

**Drug Testing in 2D hPSC Cardiomyocytes**

Apparent advantages of 2D culture conditions for drug screening are their simplicity and lower costs. Moreover, many commercially available setups are available for their analysis. Electric properties of single cells can be tested by classical (low-throughput) or automated (high-throughput) patch-clamp technology and by imaging with voltage-sensitive dyes or field potentials of 2D monolayers with multielectrode assays.67 Although principally promising, the true value of these assays still remains to be determined. A blinded study on 30 known drugs in iCells (Cellular Dynamics International) that used both field potential and Ca2⁺ transient measurements found low sensitivity and specificity values to predict human QT effects.68 Similar data were published recently by a group based on the US Food and Drug Administration comparing the effects of 20 drugs known to prolong corrected QT intervals in humans.69 Of these, 17 caused prolongations of action potentials (16 in Cor.4U [Axiogenesis] and 13 in iCell cells) and 16 of field potentials (16 in Cor.4U and 10 in iCell). Reproducing the limited sensitivity particularly of iCell cardiomyocytes. An international blinded multisite comparison is underway under the auspices of the Health and Environmental Sciences Institute to validate and extend these results.

In comparison to electrophysiological assessments, the measurement of contractile function and inotropic responses to drugs remains even more challenging in 2D. Most researchers use video-optic edge detection to determine cell motion as a surrogate of contractile force. A study testing 31 negative and positive inotropic drugs concluded that, with the exception of the β-adrenergic agonists isoprorenaline and epinephrine, positive inotropic drugs produced a response (in cell motion as detected by an IonOptix system) in the same direction as negative inotropic drugs,70 obviously limiting the usefulness of this approach. Improvements may come from advanced culture methods such as Matrigel mattresses63 demonstrating canonical positive and negative inotropic effects of calcium, verapamil, and the Ca2⁺-sensitizer EMD 570533. Of note though, isoprorenaline responses were not reported or were reversed in direction (~37% cell shortening) in long-term cultured cells,45 despite signs of advanced sarcomeric maturation (Figure 3). Under both conditions, contraction kinetics were abnormally slow (time to peak =500 ms, time to 90% relaxation =400 ms at 37°C).45 In comparison, contraction kinetics of human EHTs are much faster (100–130 ms1,72 or 100/180 ms15 for 80% contraction and relaxation times) and better reflect those determined under isometric conditions in native human heart muscles (time-to-peak force and time-to-50% relaxation 115/80 ms,72 ≈200/200 [90%] ms,73 or 165/116 [50%] ms74). One reason for the slow kinetics in 2D cardiomyocytes is likely the fact that these cells are unloaded.

**Drug Testing With 3D Engineered Heart Tissue**

Testing drugs in 3D cardiac constructs should have several advantages over testing in 2D cultured cells. As discussed above, they provide a more physiological environment for the cells and promote maturation, allow work against a defined afterload, and, importantly, the contractile and electrophysiological properties of a 3D EHT integrate the heterogeneous function of multiple (often several hundred thousands) cells. Thus, the variability between individual EHTs should be lower than that between single cells, providing advanced robustness. In the absence of direct head-to-head comparisons, much of the advantages remain theoretical at this point.

Several studies with hPSC-derived EHTs or similar 3D constructs have tested the effect of single marker drugs such as isoprorenaline at a single concentration. Although some reported a qualitatively normal, but smaller than normal (+50%) positive inotropic response,4,75 others failed to see isoprorenaline-induced changes in force at all.66,76 similar to studies in 2D cardiomyocytes. Only few studies systematically tested various drugs at different concentrations and even less in human EHTs. A study directed toward the assessment of proarrhythmic surrogates in an automated 24-well-screening platform25 with rat EHTs compared the effect on relaxation time and spontaneous rhythm of 75 drugs (33 with known proarrhythmic potential, 14 safe and 28 unselected new drugs) at 1-, 10- and 100-fold free therapeutic plasma concentrations.77 Drugs known to prolong action potential duration in rat (=inhibiting repolarization) prolonged EHT relaxation time, suggesting relaxation time as a feasible surrogate of repolarization. Not surprisingly, however, rat EHTs proved insensitive to pure Ikr blockers, consistent with the small/absent role of Ikr in rodent repolarization. In contrast, EHTs from hiPSC cardiomyocytes showed the expected prolongation of relaxation in response to 10 pure or mixed Ikr blockers at concentrations well matching hERG-IC50 values.78 Several positive and negative inotropic drugs exerted canonical, concentration-dependent responses of peak force, contraction and relaxation time at good reproducibility (Figure 4).

In this study, isoprorenaline increased the force of hiPSC EHTs by 41%, did not change contraction time, and decreased relaxation time by 20% at 2 Hz pacing rate. This compares with much larger positive inotropic (200% to 300%), clino- tropic (20%), and lusitropic (40%) effects of catecholamines in native human heart trabeculae.79,80 Nevertheless, despite the quantitatively abnormal β-adrenergic response, the data are encouraging for the use of this platform for drug testing. Recent efforts toward further simplification and miniaturization led to the fabrication of a miniaturized system to evaluate drug responses.81 Micro-Heart Muscles from <10,000 hiPSC-derived cardiomyocytes showed a positive Frank–Starling effect, and isoprorenaline increased force to a similar extent than in EHTs. Furthermore, the IC50 for verapamil was similar to the aforementioned study (970 nmol/L in EHTs versus 540 nmol/L in Micro-Heart-Muscle compared with 60 nmol/L in a 2D monolayer).81 An obvious advantage of the system is...
the low number of input cells. A disadvantage is the need to manually move the Micro-Heart tissues to an organ bath for force measurements. Another micro system supersedes this step, but only limited drug tests have been reported with this system yet. Surprisingly, when applied to microconstructs derived from neonatal rat myocytes, isoprenaline increased force at lower and decreased force at higher concentrations. More extensive blinded head-to-head comparisons between 2D and the different 3D systems are necessary to answer the question whether the larger costs (higher input of cells) in the various 3D systems compared with 2D are outweighed by better sensitivity, specificity, and robustness. Such tests are currently underway (eg, HESI-multisite comparison; Health and Environmental Sciences Institute, Washington, DC).

Disease Modeling With 3D Engineered Heart Tissue

For the reasons mentioned above, EHTs could also advance the study of disease-specific phenotypes in patient-derived hiPSC cardiomyocytes. The challenges of this approach are even greater than that for drug testing. Although drug testing assesses more or less acute effects of drug, that is, paired analyses of changes from baseline, the former compares the phenotype of different hiPSC lines or their derivatives in an unpaired analysis. Thus, the error introduced by baseline variations (eg, batch-to-batch variability in cell quality) is much larger in disease-modeling approaches than drug tests. For example, we observed that contraction kinetics of hiPSC cardiomyocytes and their respective EHTs vary greatly between different commercial providers and various proprietary hiPSC lines and between batches from the same provider (Ingra Mannhardt, unpublished data, ). Yet, all hiPSC-EHTs reacted qualitatively similar to various drugs. This inherent problem of disease modeling may be addressed by parallel testing of genetically corrected, isogenic controls, statistically relevant numbers of replicate experiments, a high degree of experimental standardization, and robust assays with low Z' factor. The relevance of these factors has been demonstrated in a recent study using an organ-on-chip approach to evaluate functional consequences of a mutation that causes the mitochondrial cardiomyopathy of Barth syndrome. It found that twitch stress can differ markedly within the same wild-type line (400–2000 Pa), but the large number of replicates (n=16–41) robustly revealed a contractile defect of cardiomyocytes in Barth syndrome (and the positive effect of a therapeutic intervention with linoleic acid). The use of isogenic lines further strengthened the findings. In other cases, mechanical or drug challenges may be required to unmask a disease phenotype. Indeed, the contractile deficit of hiPSC cardiomyocytes harboring truncating titin variants was undetectable in cardiomyocytes but readily observed in self-assembling EHTs working against the elastic resistance of silicone posts.

Cardiac Repair With Heart Muscle Patches

The idea to repair an injured heart has already been proposed >20 years ago in mice, yet a real therapeutic application seemed bold. This has significantly changed. Initial attempts have focused on mononuclear bone marrow cells. However, 15 years after the first clinical application, the therapeutic effect of these approaches is still under debate. The situation for cardiac-derived cells is similar, and the therapeutic effect is currently unknown. Recently, a prospective randomized trial tested the effect of cell-free hydrogels in patients with myocardial infarction, and the first patient was treated with a fibrin patch containing hESC-derived progenitors. Other strategies like the injection of hPSC-derived cardiomyocytes have been evaluated in large animal models, and these ideas are striving toward the clinic. Relevant remuscularization of the injured heart can be achieved with hPSC cardiomyocytes in small and large animal models, demonstrating the principal feasibility of the approach including upscaled cell production and sufficient cell survival after allogenic transplantation with clinical immunosuppression regimes.
Here, we will provide a short overview on the role of tissue engineering in remuscularizing the injured heart and refer the reader to comprehensive reviews on alternative strategies aiming at the stimulation of endogenous repair processes or the modulation of mechanical properties of the heart.

Cardiac tissue-engineering approaches need to be viewed on the background of successful attempts to remuscularize the heart by direct intramyocardial injections of cells. The latter is simple and intuitive and could probably be achieved via a percutaneous intervention. Yet, it also has disadvantages. The major problem is the low cell retention rate. Even after direct intramyocardial injection in a healthy heart, the majority of cells is washed out within the first hours after transplantation. The situation is even more complex in the injured heart. Scar tissue is less extendible than muscle, requiring higher injection pressures. It also represents a hostile environment for the transplanted cells compromising cell survival. One could argue that this problem could easily be circumvented by increasing cell numbers. However, although cardiomyocytes from hPSC are theoretically available at unlimited numbers, cell number remains a critical issue. Cardiomyocyte differentiation is still difficult, error prone (even with optimized protocols), and expensive. Moreover, increasing the cell number raises concerns about remnant pluripotent and potentially tumorogenic cells after directed differentiation. The problem may be aggravated when cells are injected in scarred tissue and a thinned ventricular wall with the chance of injection into the cavity. A second problem that has hardly been addressed to date is the functional/geometric integration of cells in the host tissue. The architecture of the heart is highly complex. On the contrary, heart muscle is composed of many other cell types besides cardiomyocytes. However, muscle bundles are aligned in a complicated manner leading to a wringing movement of the left ventricle during systole. Injected cells mature after transplantation but to date do not properly integrate. Tissue-engineering approaches bear the hope to at least partially overcome these problems, but head-to-head comparisons between the 2 strategies are sparse (2 studies are discussed below) because most groups focus either on direct cell injections or on tissue engineering thereby becoming experts on their particular strategy. Thus, possible advantages of the patch approach remain largely hypothetical to date.

Cell-Free Patches or Matrices
Cell-free approaches are attractive for their simplicity. Conceptionally, the injection of matrices with or without growth factors with angiogenic or other, less-well-defined beneficial effects on the ischemic myocardium could stimulate endogenous repair mechanisms. Materials tested include alginate and derivatives, fibrin (in this article sprayed onto the heart), decellularized heart matrices, methylcellulose and hyaluronic acid. Although many of the cited studies reported improvement of function (of injured hearts), hopes that the matrices would attract new heart muscle formation have not been confirmed. In the AUGMENT-HF trial (a randomized, controlled proof-of-concept clinical study), alginate hydrogel was injected directly into the left ventricular myocardium of 40 patients with advanced heart failure. This therapy demonstrated improvement in exercise capacity compared with standard pharmacological therapy after 6 and 12 months. However, it did not improve left ventricular function. A similar approach has used hydrogels derived from extracellular matrix from pigs and yielded positive results in a preclinical large animal model, most likely via paracrine mechanisms or mechanical support of the left ventricle. A clinical trial with porcine extracellular matrix is currently performed in the United States (NCT02305602). However, these therapeutic strategies do not target the cause of the disease, which is the loss of viable contracting myocardium, and the AUGMENT-HF trial indicates that a cell-free strategy may not be sufficient. There is, however, one exception of a cell-free patch that led to de novo cardiomyogenesis. Epicardial collagen patches containing follistatin-like 1 induced cardiomyocyte proliferation and the formation of new myocardium. This strategy improved left ventricular function after myocardial infarction in a small and large animal model after a short follow-up period and suggests that a matrix approach that immobilizes (and thereby prolongs the action of) proproliferative growth factors may in fact be a powerful therapeutic option for cardiac regeneration. Conformational trials are eagerly awaited.

Cell-Based Tissue-Engineering Strategies
Initial proof-of-principle studies were performed with patch-filled cell sheets isolated from neonatal rat cardiomyocytes, but the field has moved to hPSC-derived cells. For clinical applications, the question of the right cell type is crucial. Most groups favor either hESC-derived or hiPSC-derived cardiomyocytes. The first clinical application, however, has used hESC-derived cardiac progenitors. Currently, mainly 2 techniques are used for regenerative tissue-engineering purposes, hydrogels and stacked cell sheets.

Transplantation of hiPSC-derived cell sheets (containing 20×10⁶ cells; 45% cardiomyocytes, 5% endothelial cells, and 20% vascular cells) formed grafts after transplantation in a subacute nude rat infarct model and improved left ventricular function. However, the thickness was limited to 3 cell sheets because of hypoxia. This problem could be overcome by a stepwise surgical approach. Although scientifically elegant, repeated sheet transplantation is unlikely to be clinically applicable. The insertion of gelatin hydrogel microspheres between each cell sheet allowed the creation of thicker hESC-derived patches (≤15 sheets reaching a thickness of >1 mm) in vitro and the transplantation of constructs consisting of 5 layers in vivo. These stacked cell sheets (containing 2×10⁷ cells; 45% cardiomyocytes, 10% endothelial cells, and 40% vascular cells) survived transplantation on immune-deficient rats and improved left ventricular function for ≤3 months. One of the few studies that compared a tissue-engineering strategy to single-cell injection used the classical 3-layer approach. Transplantation of cell sheets (containing 7.2×10⁶ isolated cells from neonatal rat hearts) led to larger grafts when compared with the injection of the same amount of cells. In this study, larger graft size also led to a greater improvement in left ventricular function after myocardial infarction in a rat model. Hydrogel-based patches have been tested in different animal models and a first clinical application has been reported. Collagen- or fibrin-based patches were sutured on the
epicardial surface after injury. In a recent study, 2 collagen-based constructs (containing $2.5 \times 10^6$ cardiomyocytes each) were implanted 1 month after ischemia/reperfusion in immunodeficient rats. These constructs survived transplantation and formed stable grafts (Figure 5) but did not improve global left ventricular function in this chronic injury model.93 Our own group implanted 2 fibrin constructs (each containing $5 \times 10^6$ cardiomyocytes and $2 \times 10^6$ endothelial cells) in a subacute guinea pig injury model under pharmacological immune suppression to remuscularize the scar 1 week after injury. This strategy partially remuscularized the scar (Figure 5) and improved left ventricular function in a follow-up period of 4 weeks.94 The first implantation of a fibrin patch containing hESC-derived cardiac progenitors (characterized by the expression of Islet-1 and stage-specific embryonic antigen [SSEA-1]) was performed in a 68-year-old patient during bypass surgery. Although anecdotally to date, the physicians reported improvement of left ventricular function and clinical status after surgery.90

Cell Types and Cell Number
Human PSC now offer the opportunity to optimize the cellular composition of human heart muscle patches for transplantation. Differentiation protocols have been published for cardiomyocytes,118 fibroblasts,119 and endothelial120 and epicardial cells.119 Earlier work has shown that native endothelial cells form networks in the constructs in vitro,71 improved survival after transplantation,40,121 and participated in the vascularization after transplantation in a previous study with rat EHTs32 and cell sheets.112 It was, therefore, surprising that in our recent study with human EHTs, vascularization occurred only by ingrowth of host-derived blood vessels, and neither native human umbilical cord nor hiPSC-derived endothelial cells survived to a relevant extent.94 Inclusion of stromal cells may improve vessel stability and grafting efficiency.40

An alternative may be to prevascularize tissues in vitro, thereby allowing the generation of thicker tissues.12,55,122,123 Surgical connection of the (fragile) tissue grown in vitro to the high pressure circulation is a conceptional problem of the prevascularization approach but may be solved by incorporating native blood vessel circuits in the growing tissue23 or by the AngioChip approach.12 Future work needs to determine whether the higher regulatory hurdles of these complex approaches are outweighed by better transplantation results.

Cell number/graft size is another issue that has not been evaluated in detail to date. A meaningful comparison of cell numbers from different studies is difficult because cell composition and purity after directed differentiation vary widely. Yet, reported cell numbers in cardiac patch studies that resulted in the formation of comparable graft sizes in small animal models were considerably lower ($2.5–14 \times 10^6$ cells)93,94 than

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**Figure 5.** Results after remuscularization with hPSC-derived engineered heart tissue (EHT), cell sheets or cardiomyocytes. Masson-trichrome staining of a rat heart after transplantation of hESC-derived EHTs (A, bar=5 mm). Dystrophin (brown; low magnification) and human Ku80 staining (red; high magnification) of a short-axis cross-section 4 wk after transplantation of 2 human induced pluripotent stem cell (hiPSC)-derived EHTs in a guinea pig model (B, bar=1 mm). Troponin T (green) and human nuclear antigen staining (red) of a rat heart after transplantation of stacked cell sheets derived from hiPSC cardiomyocytes (C, bar=2 mm). Results after direct cell injections in nonhuman primates with hESC-derived cardiomyocytes in (D; bar=2 mm) and hiPSC-derived cardiomyocytes in (E, bar=1 mm). Cardiomyocytes are GFP (green-fluorescent protein)-labeled (green). A, Reprinted from Riegler et al93 with permission of the publisher. Copyright © 2015, American Heart Association. B, Reprinted from Weinberger et al94 with permission of the publisher. Copyright © 2014, The American Association for the Advancement of Science. C, Reprinted from Masumoto et al114 with permission. Copyright © 2014, The Authors. D, Reprinted from Chong et al96 with permission of the publisher. Copyright © 2014, Nature Publishing Group. E, Reprinted from Shiba et al95 with permission of the publisher. Copyright © 2016, Nature Publishing Group.
with cell injections (10–100×10⁶ cells92,125), compatible with the low retention rate after cell injection. The percentage of grafted cells (as a result of cell proliferation and cell death) appeared similar with collagen and fibrin constructs.93,94 A systematic analysis of the minimal effective dose (i.e., the minimal effective cell number) is necessary but likely difficult to assess in small animal models for technical reasons. This question is especially interesting because no correlation between graft size and functional recovery has been documented to date.

**Cell Survival and Proliferation**

Human PSC cardiomyocytes are immature and therefore resistant to hypoxia. Own experiments showed that human EHTs are able to function without an apparent loss of function under anoxic conditions, at least for an hour. However, hPSC cardiomyocytes mature over time in the EHT format.75,78 Although maturation increases force production, it may also decrease resistance to hypoxia. It is well established that adult myocytes do not survive transplantation.125 Immature cells possess the ability to proliferate, and net remuscularization is the result of cardiomyocyte death and washout on the one hand and cardiomyocyte proliferation on the other hand. In the guinea pig study cited above, 2 strip-like hiPSC EHTs with a diameter of 1.5 mm (length 18 mm) remuscularized a mean of 12% of the scar area and gave rise to human muscle grafts with a diameter of ≤1.5×4 mm.98 Thus, in some cases, the new muscle structures were significantly larger than the input human EHTs. Cardiomyocyte proliferation is difficult to assess in a normal heart.126 It is even more difficult to exactly assess proliferation of the xenogenic approach as allogenic transplantation (with cells injected directly into the myocardium. Epicardial transplantation induces an injury response that is characterized by a fibrinous cap physically separating the patches from the heart.94,101 The fibrotic response may be partially because of the xenogenic approach as allogenic transplantation (with neonatal rat cardiomyocytes) seemed to induce less scarring.32 It is questionable whether suturing patches to the healthy border zone myocardium can fully overcome this problem. A study compared the epicardial approach with direct intramyocardial injection of cardiomyocytes and microtissue particles. Epicardial transplantation led to large grafts that, in contrast to microtissue particles and single cell injections, did not couple to the host myocardium.103 Our group demonstrated that EHTs are principally able to electrically couple to the injured host myocardium.9 Yet, coupling was observed only in a subset of hearts, and it is not known whether it was the consequence of direct myocyte–myocyte connections or happened via fibroblasts. Evaluation of electric coupling to the host myocardium is technically challenging. Genetically modified cells expressing calcium sensors such as GCAMp (genetically encoded calcium indicator) represent the gold standard for the evaluation of electric coupling. Approaches that rely on the application of voltage-sensitive dyes can fail because they may not adequately label transplanted cells when perfused via the coronary arteries.92 An alternative is the epicardial application of a voltage-sensitive dye.6 Voltage-sensitive dyes or transgenic voltage sensors127 may be good tools to analyze action potential propagation within the transplanted cells or constructs.

**Electric Coupling**

Electric coupling of the patch to the host myocardium is an essential component for active participation to the contraction and may be more critical with an epicardial patch than with cells injected directly into the myocardium. Epicardial transplantation induces an injury response that is characterized by a fibrinous cap physically separating the patches from the heart.94,101 The fibrotic response may be partially because of the xenogenic approach as allogenic transplantation (with neonatal rat cardiomyocytes) seemed to induce less scarring.32 It is questionable whether suturing patches to the healthy border zone myocardium can fully overcome this problem. A study compared the epicardial approach with direct intramyocardial injection of cardiomyocytes and microtissue particles. Epicardial transplantation led to large grafts that, in contrast to microtissue particles and single cell injections, did not couple to the host myocardium.103 Our group demonstrated that EHTs are principally able to electrically couple to the injured host myocardium.9 Yet, coupling was observed only in a subset of hearts, and it is not known whether it was the consequence of direct myocyte–myocyte connections or happened via fibroblasts. Evaluation of electric coupling to the host myocardium is technically challenging. Genetically modified cells expressing calcium sensors such as GCAMp (genetically encoded calcium indicator) represent the gold standard for the evaluation of electric coupling. Approaches that rely on the application of voltage-sensitive dyes can fail because they may not adequately label transplanted cells when perfused via the coronary arteries.92 An alternative is the epicardial application of a voltage-sensitive dye.6 Voltage-sensitive dyes or transgenic voltage sensors127 may be good tools to analyze action potential propagation within the transplanted cells or constructs.

**Target Population for Cardiac Tissue Patches**

As clinical application seems principally achievable, new, to date undiscussed, questions occur. One of them is the selection of the right patient population. Most studies currently target ischemic heart disease. However, dilated cardiomyopathy or congenital heart defects might also represent possible targets for regenerative strategies with tissue-engineered constructs. Interestingly, the time point of transplantation seems to play a major role. The transplantation of cells or tissue-engineered patches seems to be more successful when it is performed in a subacute myocardial injury setting.92,94 Chronic injury models, which more closely resemble the situation in most advanced heart failure patients, seemed to be less accessible for regenerative strategies.93,128 This has not been addressed systematically but may represent a major hurdle for clinical applications. A second important question deals with immune suppression. Initial clinical applications with hESC or hiPSC will be allogenic. It has been demonstrated that allogenic transplantation of hiPSC-derived cardiomyocytes can be successfully performed with tacrolimus and methylprednisolone in clinically acceptable doses.95 Transplanted cell sheets without immunosuppression, in contrast, were rejected even in major histocompatibility complex–matched nonhuman primates.129 This finding is not surprising, but therapeutic application then comes with the typical side effects of long-term immunosuppression. Unfortunately, immune suppression might also preclude patients on left ventricular assist device (an otherwise ideal patient population) because these patients already possess a high risk for lethal infections.130 Choosing the right patients for first-in-man trials, therefore, will be of utmost importance.

**Summary and Outlook**

Cardiac tissue engineering represents a maturing field, and relevant progress has been made during the past years. It is possible to create cardiac tissue from human stem cells that closely resemble the native heart. We, therefore, think that tissue engineering might fulfill at least some of the proposed expectations in the near future. We have discussed 3 possible applications for cardiac tissue engineering, namely, drug screening, disease modeling, and cardiac repair. In all of these fields, single cells are strong competitors. 3D constructs provide a higher content and more physiological condition that, by integrating the function of numerous individual cells, provide a more stable readout than 2D cells for drug screening and disease modeling. However, these advantages have to be proven by direct head-to-head comparison. These are not the most sophisticated and scientifically rewarding experiments but are critical to move the field forward toward an industrial application. Cooperation between different laboratories
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