Physiological aspects of cardiac tissue engineering

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Eschenhagen T, Eder A, Vollert I, Hansen A. Physiological aspects of cardiac tissue engineering. Am J Physiol Heart Circ Physiol 303: H133–H143, 2012. First published May 11, 2012; doi:10.1152/ajpheart.00007.2012.—Cardiac tissue engineering aims at repairing the diseased heart and developing cardiac tissues for basic research and predictive toxicology applications. Since the first description of engineered heart tissue 15 years ago, major development steps were directed toward these three goals. Technical innovations led to improved three-dimensional cardiac tissue structure and near physiological contractile force development. Automation and standardization allow medium throughput screening. Larger constructs composed of many small engineered heart tissues or stacked cell sheet tissues were tested for cardiac repair and were associated with functional improvements in rats. Whether these approaches can be simply transferred to larger animals or the human patients remains to be tested. The availability of an unrestricted human cardiac myocyte cell source from human embryonic stem cells or human-induced pluripotent stem cells is a major breakthrough. This review summarizes current tissue engineering techniques with their strengths and limitations and possible future applications.

embryonic stem cells; induced pluripotent stem cells; heart; disease modeling; force of contraction

Introduction

Tissue engineering holds two promises: to generate surrogate three-dimensional (3-D) tissues for organ repair and experimental test beds modeling physiological organ function better than standard two-dimensional (2-D) monolayer cell cultures. A quarter century after coining the term “tissue engineering,” initial overoptimistic expectations have made way to a more realistic appraisal of its chances and limitations (47). Here we review the current state of myocardial tissue engineering for cardiac repair, preclinical drug development, and disease modeling.

Tissue Engineering for Cardiac Repair: Different Approaches

Heart muscle cells lose their capacity to divide early after birth (85), which makes their loss due to myocardial infarction, increased hemodynamic load, infection, genetic disorders, or toxins essentially irreversible. Whether the loss of myocytes is indeed complete or residual regeneration is retained is an area of active debate (3, 7, 38, 51, 59, 85), but there is no doubt that loss of a relevant fraction of myocytes (e.g., during myocardial infarction) leads to a permanent reduction in contractile function and eventually heart failure, a growing medical problem worldwide. It is intuitively appealing, therefore, to substitute the loss of myocytes by exogenous or endogenous sources, the promise of “regenerative cardiology” (18, 63, 75, 86). In this scenario, engineering techniques could play different roles.

The simplest and potentially most straightforward engineering technique is to create conditions in the (patient) heart that promote cardiac myocyte progenitor cell homing to the (injured) myocardium (in situ tissue engineering). Examples are implantation of nanofibers (15), alginate (48), or cardiac extracellular matrix scaffolds (29). The attraction of this approach is that it is simple, can easily be done under good manufacturing practice and good clinical practice conditions, and can be marketed. Indeed, the first clinical studies with alginate injections after myocardial infarction are underway in Europe and Israel (clinicaltrials.gov, NCT01226563, NCT01311791). The big question, however, is whether cardiac progenitors exist in the adult heart at all (particularly in elderly patients) and, if yes, whether their homing and tissue building capacity can be promoted by injecting extracellular matrices or fibers. If not, the approach could still promote angiogenesis and/or homing of nonmyocytes that, via paracrine factors, improve the survival of surrounding myocytes. None of these questions has been unambiguously answered, but a recent publication using an elegant genetic approach concluded that injected hematopoietic cells do not transdifferentiate into myocytes as initially suggested (63) but stimulate an (unidentified) endogenous progenitor population to form new myocardium after experimentally induced infarction in mice (51).
The second engineering approach aims at increasing the efficiency of (stem) cell application to the injured heart. Simple cell injection into the beating myocardium leads to massive rates of immediate cell death and very low retention rates over time. Carefully performed studies report that values of <10% of cells remain a few days after cell injection in the heart of animals (19, 69, 101) or intracoronary infusion in patients (36). When using potentially tumorigenic cells such as embryonic stem cells, the high washout rate not only reduces their potential therapeutic efficiency but also leads to cell deposition in the lung, liver, kidney, and spleen (19) and constitutes a clear risk of systemic tumor formation as demonstrated in mice (11).

Tissue engineering methods can improve cell retention rates. Probably the most advanced approach in this respect is the cell sheet technique (62, 83). Key to this technique was the development of thermosensitive culture dish surfaces that allow detachment of intact cellular monolayers (of principally unlimited size in 2-D) at room temperature. The resulting thin sheets can be directly attached to the beating heart without glue or sutures. The group has developed semiautomated devices to facilitate and standardize this procedure for clinical application and reported the first implantation of cell sheets in a patient with severe dilated cardiomyopathy (78). Despite the use of autologous skeletal myoblasts that lack the capacity to differentiate into cardiac myocytes and do not couple to the host myocardium (74), improvements of cardiac function were reported. This and former studies with mesenchymal stroma cells (53) or adipocytes (40) support the idea that the effects of the cell sheet technique are, at least to a large part, due to paracrine activity and mechanical stabilization of the scarred ventricle (33). Advantages of the cell sheet technique are its simplicity, the selection against dead cells in the preimplantation step in vitro, and very high cellular retention rate. Cell sheets can also be stacked to form thicker tissues. Unfortunately, however, this possibility is, in the case of neonatal rat heart cells, limited to 3 to 4 sheets (30–40 μm total thickness) by the natural diffusion limits of factors necessary for and released by metabolically active myocytes. A multistep surgical procedure in which cell sheets were stacked on each other repeatedly with intervals sufficient to allow vascularization overcame the limitation (82), but it is unlikely that a multistep open-heart surgery could be transferred to patients. Alternative ideas to increase cell retention in a cell therapy approach include injection of cells in collagen, matrigel, fibrin, or other extracellular matrices that form hydrogels (1, 14, 46). Whereas the hydrogel certainly helps to retain cells in the area of injection, it is less clear whether (and if yes, how) it can overcome the high death rates induced by the pressure needed to inject cells in a relatively stiff environment such as the beating ventricular myocardium.

The third “traditional” tissue engineering technique aims at building functional 3-D artificial heart tissues that can be implanted onto the injured heart. Apparently, this is the most ambitious and difficult goal and has been approached from three different angles. One is to try to fabricate matrixes that imitate the natural extracellular environment as closely as possible so that cells can integrate into it and form a tissue. Examples of prefabricated, solid matrixes are alginate (49), polyactic acid (65), polyglycolic acid (66), and mixtures thereof or collagen sponges (72). The great advantage of this approach is the principally unlimited possibility to vary shape, size, and modulus of the construct. To date, however, the ability to imitate the natural extracellular environment and the microstructure of the heart remains imperfect at best. Moreover, a stereotypic observation with various solid matrixes is that cells do not easily enter the matrix and distribute in free spaces. Instead, they essentially seed on the surface and a few micrometers beyond (49, 65, 72). Related to this issue is that prefabricated porous matrixes prevent rather than promote cell-cell contact by separating cells via matrix septa. Coupling of cells is an essential requirement for tissue formation, and its prevention by matrixes thus remains an unresolved conceptual problem. A second matrix-based tissue engineering approach replaces technical engineering of a nature-like matrix by de-cellularizing intact heart tissues, e.g., from pig hearts (64). A relatively simple sodium dodecyl sulphate- and Triton X-100-based procedure allows complete removal of the heart’s cellular components while preserving a surprisingly intact natural matrix of both the myocardium and great blood vessels (64). Whereas this approach elegantly solves the question of how to obtain a nature-like matrix, it does not overcome the inherent problems of having cells quantitatively enter the matrix. A possible route could be provided by the intact blood vessels, but here the advantage of preservation apparently turns into a disadvantage, because cells when perfused into the (decellularized) coronary arteries did not quantitatively egress from the circulation and invade the surrounding myocardial matrix (64). Thus the dream of the complete, vascularized artificial heart will likely remain unfulfilled for a while, but it will be interesting to see how this issue can be solved. Third, a conceptionally different matrix-based approach employs liquid hydrogels such as collagen I (8, 22, 110), matrigel (55), or fibrin to make tissues ex vivo (34). Here freshly isolated cardiac cells are mixed with natural, gel-forming, extracellular matrixes that fulfill the main purpose of keeping cells in a 3-D space until they form cell-cell contacts and generate their own extracellular matrix, thereby forming a tissue. The hydrogels also appear to protect isolated cells from a process termed anoikis, cell death due to loss of cell-cell contacts (41, 57). In the various hydrogel approaches currently pursued, the macroscopic form of the construct is defined by respective casting molds and the techniques to fix the developing tissue to holders (Fig. 1). The latter is crucial because good cardiac tissue development occurs only in constructs growing under continuous mechanical load (8, 22, 26, 109), an observation also made with engineered skeletal muscles (96–97). Strain on the developing tissue derives from cells forming contacts to neighboring cells and remodeling the extracellular matrix, which leads to retraction of the hydrogel and tension development if the ends are fixed to something. In the absence of mechanical strain, cells degenerate and do not form a beating tissue (own unpublished observation); in its presence a near physiological heart tissue development occurs (Fig. 2). The heart consists of fibroblasts, endothelial cells, and cardiomyocytes, raising the question as to whether a certain ratio of these three cell populations supports constitution of cardiac tissues in vitro. A few reports have addressed this question systematically and most support the hypothesis that endothelial cells and fibroblasts support tissue development (12, 60, 71, 94), but one also reported inferior outcomes in the presence of fibroblasts (50), indicating that more work is required to answer this question.
Cardiac Repair with Engineered Tissues: Results from Animal Experiments

Numerous studies tested engineered tissues or related products in small series of standard laboratory animals. Moreover, composite cell sheets made from adipocytes and embryonic stem cell-derived cardiovascular progenitors were implanted in five infarcted nonhuman primates (6), and recently, the first patient was treated with an autologous myoblast cell sheet (78). All studies reported some functional benefit. However, studies are lacking that compared the functional consequences of the different cardiac tissue engineering approaches described above systematically and in a statistically meaningful way. Noncardiac cells (adipocytes) were applied as sheets or suspended cell injections in a direct comparison, showing better results with the sheet technique (33). However, here the mechanism can only be paracrine or mechanic, a precluding conclusion on cardiac constructs. Thus, at this point, it is impossible to substantiate the widely held belief that tissue engineering provides a better means to repair a heart than direct application of cells or that one technique is superior to another. Yet, the published studies provide a number of important messages.

First, despite the lack of immediate vascularization (see below), thin cell sheets (28, 82) and collagen I/matrigel-based engineered heart tissue (EHT) (106, 109) survived when implanted in immunodeficient/-suppressed rats/mice (both on the heart and extracardiac places) and formed new, relatively well-organized heart tissue. The quality and thickness of the new tissue was much better than anything published to date with injected cells. EHTs from neonatal rat hearts induce strong immune responses that need to be suppressed to preserve the tissue after implantation (21, 106). Interestingly,
EHTs made from unfractionated neonatal rat hearts develop vascular structures in vitro that appear to participate in the formation of blood-perfused vasculature after implantation onto the left ventricle (60, 81, 109). Similar findings were made in stacked cell sheets from neonatal rat hearts (81) and EHTs from human embryonic stem cell (hESC)-derived cardiac myocytes that were mixed with endothelial cells (12, 94). Vascularization was extensive a few weeks after implantation onto the heart (21, 81, 106, 109). The exact time course remains insufficiently studied.

Second, implanted engineered tissues electrically connect to the host heart (28, 109). This may seem trivial, but it is all but that. On the one hand, the tissues are implanted on the pericardial surface of the heart and, in case of EHTs, contain a continuous layer of epithelial-like cells on the outer surface. Thus at least two nonmyocyte layers separate host and donor myocytes from each other at the time of implantation. And these layers appear to increase in thickness rather than disappear over time, because an apparently isolating fibrous layer was consistently observed between the implant and the host myocardium a few weeks after implantation. So how does coupling occur? Extensive direct myocyte-to-myocyte coupling has never been observed by histological means, but some “bridge-like” connections have been reported (106). Whether they can account for the almost undelayed electrical conductance between host and donor tissue (28, 109) is unclear. An alternative could be “electrotonic coupling” between host and donor myocardium, which occurs within ≈1 length constant (0.15 mm to 0.3 mm) over the entire surface (23).

Third, implantation of multiloop EHTs (by fusion of five ring-format EHTs) improved diastolic and systolic function in infarcted rodent hearts (109). The effects of implanting large multi-loop EHT on cardiac function were substantial (increase in fractional area shortening by 30%) and included re-establishment of wall thickening of the scar at the implantation site in systole, a sign of new vital myocardium. These magnetic resonance imaging results were associated with the formation of a layer of new myocardium at the implantation site (0.5–1 mm in histological sections), enough to explain the functional improvement. Furthermore, control EHTs made from noncardiac myocytes or nonvital EHTs did not have the same effect. The study can therefore serve as a proof of principle for the therapeutic potential of myocardial tissue engineering. Yet, others described similar functional consequences using noncardiac cell sheets (33, 40, 53, 80) without clear evidence for the formation of new myocardium. Thus the mechanisms which accounts for functional improvements after implanting cardiac or noncardiac tissues onto the heart remain to be fully deciphered.

Recent Improvements in Cardiac Tissue Engineering

For many years, myocardial tissue engineering was restricted to embryonic chicken and neonatal rat or mouse heart cells, simply because a source for human cardiac myocytes was unavailable. This has changed dramatically because of the recent progress in stem cell biology. In 1998, the first hESCs were described (92), and three years later cardiac myocytes were generated from hESCs (44). hESCs provided, for the first time, an unlimited cell source with an undisputed capacity to differentiate into essentially all types of cells of the body including cardiac myocytes. Their discovery boosted cardiac tissue engineering by answering the question as to where the several hundred millions of human cardiac myocytes that are necessary to make large myocardial patches may come from (107). The ethical concerns with hESC work stimulated the search for pluripotent alternatives. These combined with the technical experience acquired with mouse and hESC cultures were important drivers of the seminal discovery of methods to induce pluripotency in mouse and human somatic cells by introducing a combination of pluripotency factors (89).

hESCs, at least in principle, solved the issue of a renewable human cardiac myocyte source for cardiac regeneration and human-induced pluripotent stem (hiPS) cells that of a patient-derived autologous approach. Another important bottleneck remained for years, the low efficiency of cardiac myocyte differentiation from pluripotent stem cells. This problem was solved by studies that carefully deciphered the factors that
guide the earliest steps of cardiac development under normal conditions and translated this knowledge into cell culture protocols [e.g., (56)]. Recent multistep protocols with growth factors that sequentially drive mesodermal and cardiac specification increased cardiac myocyte differentiation rates from ~1% (44) to 50% or more (100). By optimization of growth factor combinations and timing, cardiac myocyte differentiation rates can be increased even further and, importantly, the new protocols can be applied to essentially all pluripotent stem cell lines including hiPS (42).

The progress in stem cell technology was crucial for the engineering of human heart muscle for several reasons. First, the high purity of cardiac myocytes allowed human EHT to be generated without further purification (79, 94). This has obviated the need for manual dissection of beating clusters (12) or genetic selection (2) with their inherent limitations. It has also improved tissue homogeneity compared with the early work with hESC-derived cells (12–13, 87). Second, just as important, the higher cardiac myocyte yield has allowed routine work with engineered human heart muscles, opening new in vitro applications (see below). Third, perspective, the high efficiency of growth factor-based differentiation protocols shall substantially reduce the risk of teratoma formation by driving pluripotent cells quantitatively into the mesenchymal or cardiac lineage (see below). More work is needed to substantiate this assumption.

Open Questions on the Way to Clinical Application

1) Are tissue engineering approaches with actively contracting tissues really superior to simple cell injections/infusions, transplantation of noncontracting tissues or standard medical therapy? Head-to-head comparisons under well-controlled conditions are needed to answer these questions. Such studies will require randomization, accurate and robust readouts, blinded investigators, and large numbers of animals.

2) What is the optimal animal model to assess this question? Differences in cardiac physiology and size make mice and rats principally suboptimal models. Thus it seems unlikely that human EHTs (which finally should be tested) can stably couple to a heart that beats at 600 (mouse) or 300 (rat) beats/min, respectively. A possibility is to transplant human EHT into immunosuppressed primates, but for ethical and economic reasons such experiments require careful exploitation of all other options. For example, EHTs could be generated from larger and clinically more relevant animals such as (neonatal) rabbits, pigs, or dogs to be transplanted in an allogeneic procedure. Nobody has yet produced tissues from these species and own attempts with newborn pigs were unsuccessful (unpublished data). However, porcine iPS cells have been recently established (24) and could be a good future source of allogeneic cardiac myocytes for clinically relevant preclinical studies in pigs with heart failure.

3) Do recent growth factor-mediated cardiac differentiation protocols completely eliminate pluripotent stem cells and thereby the risk of teratoma formation of engineered myocardial tissues? Rigorous long-term experiments with a suitable number of animals (immunodeficient mice, rats) are needed. Recent antibody-based selection protocols may be helpful in this respect (20).

4) How to drive hESC/hiPS cell-derived myocytes and EHTs into advanced maturation? The 3-D environment per se, mechanical loading and electrical stimulation have been identified as factors promoting maturation, but even in EHT or similar constructs cardiac myocytes remain immature (79, 94). Recent evidence suggests that neuregulin may improve ventricular specification (104), but more work is clearly needed in this area.

5) Does the relatively high risk of chromosomal aberrations, gene duplications, and mutations in iPS cell lines (30, 39) affect the function of (terminally differentiated) iPS-derived cardiac products and the outcome of implantation studies?

6) Are cardiac myocytes/tissues from autologous iPS cells devoid of immune responses? Recent data suggest that this does not need to be the case (102), but this may depend on the type of differentiated cell. This is challenging in practice to study because iPS cell lines would need to be generated from animals that, several months later, would serve as their own recipients.

7) An open question related to the previous is, Which of the three pluripotent stem cell sources are best suited for clinical applications? IPS cell products have the theoretical advantage of being autologous. But besides this (still unproven) advantage, logistic factors rather argue against an autologous and for an “off the shelf” approach. Specifically, the time needed to generate iPS-derived cardiac tissues amounts to a minimum of 4–6 mo, potentially too long for patients suffering from a severe myocardial infarction. Parthenogenetic stem cells (76) have theoretical advantages in terms of antigenic heterogeneity, which would reduce the number needed for stem cell banks (105).

8) Is it possible to produce large-enough tissues that have a realistic chance to support the contractile function of the injured human heart (wall thickness > 10 mm)? Present engineered myocardial tissues do not exceed a thickness of 50–200 μm in vitro because of the absence of perforation and biological diffusion limits (34, 70, 73, 82, 106). One possible approach is to implant loose 3-D networks of relatively immature cells that, after connection to the host circulation will hypertrophy, thereby increase tissue density, as observed in a study with multiloop EHTs (109). An alternative is to develop methods to integrate functional blood vessels into 3-D cardiac constructs, perfuse them in vitro, and connect the “vascularized” cardiac tissue to the host circulation. Materials created using 3-D printing techniques (61) and decellularized natural vascular beds (29) are at hand. An elegant alternative in vivo prevascularization approach has been described recently (55). Neonatal rat heart cells were mixed with matrigel and poured into a chamber implanted close to an atrioventricular loop that had been surgically constructed from the epigastric artery and vein (55). This led to the formation of a well-developed, thick (up to 2 mm) cardiac tissue with an autologous vascular bed, suitable for implantation and connection to the host circulation.

9) Can engineered cardiac tissue be induced to integrate correctly into the complex architecture of the heart? In contrast to skeletal muscles in which fibers are strictly longitudinally oriented, heart muscle strands rather form flat bands that are wrapped around a cavity and can be oriented perpendicular to each other in different layers. Theoretically, such architecture could be mimicked using a completely synthetic matrix-based approach (e.g., 3-D printing) or decellularized hearts, but it
remains unclear whether cells applied to such constructs would automatically follow the scaffold orientation. Hydrogel-based methods cannot mimic the complex heart architecture a priori but, when supplying cells in the gel with a directed mechanical load, induce a strict longitudinal tissue orientation (34, 110). Whether this orientation will remain after implanting the tissues on the heart or adapt to new strain lines of the host environment remains unknown.

10) Could a cardiac patch be fixed onto the endocardium (which is the first tissue to be affected by ischemia) rather than the epicardium? And would it yield better results despite the clearly more invasive technique?

Tissue Engineering to Model (Human) Heart Tissue Function

Whereas cardiac regeneration may be the more exciting goal, modeling heart tissue function in the dish is probably a more realistic and short-term application of engineered cardiac tissues. Indeed, we believe that 3-D cardiac tissues will soon play a role in preclinical drug development and iPS cell-mediated disease modeling. The main promise of the in vitro approach was defined in the first tissue engineering conference in 1987 (http://www.nsf.gov/pubs/2004/nsf0450/emergence.pdf) and, specifically for the cardiac field, 15 years ago (22). Engineered 3-D tissues should mimic the natural cell environment better than 2-D cultures. Theoretical advantages include the following. First, cells in a 3-D tissue can communicate and make contacts with surrounding cells in all directions. In contrast, cells in standard monolayer cell cultures sit on a rigid plastic surface and have only side-to-side contacts with neighboring cells. Second, cells in 3-D form an organized structure and proliferate less, which allows for long-term studies and alleviates the need for pharmacological suppression of mitotic activity as frequently done in 2-D cultures. Third, the 3-D environment may promote differentiation of cardiac myocytes toward a fully mature phenotype relative to 2-D. Evidence supporting this hypothesis exists (93), but studies with hESC- or hiPS-derived myocytes also show that 3-D alone is not the cue for terminal maturation (79, 94). Fourth, contractile function measurements can be made more accurately in 3-D than in 2-D cultures. The initial hope was to establish 3-D engineered cardiac tissues as an experimental model placed between standard 2-D cultures and more complex experimental cardiac systems such as Purkinje fibers, papillary muscles/trabeculae, Langendorff-perfused hearts, or the entire animal. The following paragraphs summarize how much of the promise of 3-D tissues as models for cardiac muscle function has been fulfilled. Almost all data were derived from experiments using the hydrogel approach, which, in contrast to other tissue engineering techniques, has been designed for functional measurements of 3-D cardiac tissues in vitro and has found widespread application. Over the years, the original lattice approach (22) was modified and developed toward an improved in vitro assay by modifying cell source, casting molds, cellular composition, mode of stretching, force measurement, etc. (Fig. 1). Condensation of the hydrogel and degradation of the exogenous extracellular matrix are essential aspects of the formation of a densely packed cardiac tissue in vitro (Fig. 2). The mechanisms underlying this process are poorly understood and need to be studied systematically.

Force development. The fundamental function of the heart is the development of contractile force. Based on single cell analysis human ventricular cardiomyocytes develop a force of 50 mN/mm² under optimized preload (95). Intact human trabeculae in an organ bath usually develop between 1 and 10 mN, which, if very thin, well-oxygenated muscles are used, amounts to similar relative values [e.g., 44 mN/mm² (35)]. Tissue-engineered muscles, in contrast, generally develop much smaller forces, ranging between 0.1 and 4 mN/mm² (Table 1). One reason is that the fraction of the engineered cardiac construct populated by cells and compact muscle strands is generally much smaller than in normal hearts. The rest is cell-free extracellular matrix, which does not contribute to contractile force but to tissue diameter (denominator) and to passive stiffness. In consequence, active/passive force ratios are often smaller in large engineered tissues (Table 1) than values reported from nonfailing, ischemic and dilated cardiomyopathy heart preparations, amounting to 1–2 (37, 98). Near-physiological force values have only been measured in ultra-thin constructs (5) or aged EHTs almost devoid of free extracellular matrix (34). Inhomogenous cell distribution and low cellular density in engineered heart constructs are due to the fact that cells in engineered 3-D tissues rely exclusively on diffusion as the supply of nutrients and oxygen, which is only sufficient within the most peripheral 50–100 μm of a tissue or a muscle strand inside a tissue. Thus thicker constructs, particularly those made from preformed matrices, show a steep gradient of cell density from the surface to the core (22, 73, 87, 94). The conclusion from these findings is the contractile force of engineered cardiac constructs can approach physiological values if constructs are sufficiently small and extracellular matrix content low but does not simply increase with the size of the construct. Another reason for subnormal forces reported in many studies is that, in the absence of stretch or other means to direct cells, cells are isotropically oriented and thus do not transmit their contractile force in a coordinate manner. Force measurements in such constructs are therefore relatively meaningless. The immaturity of cardiac myocytes likely also accounts for lower force development. In normal adult cardiac myocytes with their almost crystalline organization the myofilament:mitochondria:nucleus ratio is 47:36:2 with the remaining consisting of 3.5% sarcoplasmic reticulum and 11.5% cytosol (43). In myocytes visualized in sections from rat EHTs by transmission electron microscopy, the ratio was 45:24:9 with 23% for sarcoplasmic reticulum and cytosol (110). These values have not been quantitatively determined in constructs from hESCs or hiPS cells but are considerably lower, indicating the low degree of maturity.

Reaction to physiological interventions. Cardiomyocytes, similar to skeletal muscle cells, increase active force development in response to increased preload (Frank-Starling mechanism). Several studies have shown the same to be true in engineered cardiac constructs (Table 1 and (4, 26, 50, 94, 108, 110)) and reported increases between 1.9–10-fold. The substantial variation is likely in part due to practical difficulties in defining baseline values for force development. A second physiological response is the force-frequency relationship, in which a normal human heart muscle increases force with increasing stimulation rate (Bowditch phenomenon 1871, http://vlp.mpiwg-berlin.mpg.de/library/data/lit1387/index_html?p=1&ws=1.5). Engineered cardiac tissues show the same response, albeit at different directions (Table 1).
Whereas chicken EHTs show a positive staircase (between 0.8 and 2 Hz), EHTs from rat and mouse exhibit a negative correlation between 1 and 5 Hz. Myocytes also increase their contraction kinetics with increasing frequency, a phenomenon called frequency-dependent acceleration of relaxation (17). One report in mouse EHTs did not observe frequency-dependent acceleration of relaxation between 6 and 8 Hz (16), but systematic studies over larger ranges of frequency are lacking.

**Reaction to pharmacological interventions.** Cardiac, in contrast to skeletal, muscle is exquisitely depending on extracellular Ca\(^{2+}\)-concentrations. A number of tissue engineering approaches analyzed the relationship between force development and changes in extracellular Ca\(^{2+}\) concentration (22, 26, 68, 79, 108–110). These experiments demonstrate that engineered neonatal rat cardiac tissues respond to changes in extracellular Ca\(^{2+}\) similar to intact cardiac tissues of the rat. However, a frequent observation in these studies was a hypersensitivity to extracellular Ca\(^{2+}\), i.e., a leftward shift of the force-Ca\(^{2+}\) relationship compared with adult rat heart preparations. One factor accounting for this observation may be the developmental stage of engineered cardiac constructs. Whereas one study in neonatal rat heart preparations did not show Ca\(^{2+}\) hypersensitivity (77), others observed a marked leftward shift of the force-Ca\(^{2+}\) relationship in skinned myofibers from embryonic (embryonic day 16.5) and fetal mouse (embryonic day 19.5) hearts compared with neonatal (postnatal day 7), or, even more pronounced, to adult mouse hearts [postnatal weeks 6–8 (84)]. These findings were associated with differences in the expression pattern of sarcomeric proteins, indicating that myofilament composition contributes to higher Ca\(^{2+}\) sensitivity in the immature heart. Additionally, the relative importance of sarcoplasmic reticulum and sarcolemmal Ca\(^{2+}\) cycling.

### Table 1. Physiological and pharmacological features of engineered cardiac constructs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study</th>
<th>Species</th>
<th>Quantification</th>
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<tr>
<td><strong>Active force/diameter</strong></td>
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<td>Rat</td>
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<td>Rat</td>
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<td>Rat</td>
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<td>Rat</td>
<td>28.7 mN/mm(^2)</td>
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<td>Boudou et al., 2012 (9)</td>
<td>Rat</td>
<td>0.96 mN/mm(^2)</td>
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<td>Asnes et al., 2006 (4)</td>
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<td>0.91 mN/mm(^2)</td>
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<td></td>
<td>de Lange et al., 2011 (16)</td>
<td>Mouse</td>
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<td></td>
<td>Tulloch et al., 2011 (94)</td>
<td>Human</td>
<td>0.08 mN/mm(^2)</td>
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<td><strong>Force (active/passive/ratio)</strong></td>
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<td>de Lange et al., 2011 (16)</td>
<td>Mouse</td>
<td>1 mN/1.1 mN/0.9</td>
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<td>Tulloch et al., 2011 (94)</td>
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<td>Rat</td>
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<td></td>
<td>Lai et al., 2011 (50)</td>
<td>Mouse</td>
<td>1.9-fold</td>
</tr>
<tr>
<td></td>
<td>de Lange et al., 2011 (16)</td>
<td>Mouse</td>
<td>10-fold</td>
</tr>
<tr>
<td></td>
<td>Tulloch et al., 2011 (94)</td>
<td>Human</td>
<td>8-fold</td>
</tr>
<tr>
<td><strong>Isoprenaline</strong></td>
<td>Fink et al., 2000 (26)</td>
<td>Rat</td>
<td>ND, 1.19-fold</td>
</tr>
<tr>
<td></td>
<td>Zimmermann et al., 2002 (110)</td>
<td>Rat</td>
<td>3 nM, 2.5 fold</td>
</tr>
<tr>
<td></td>
<td>Münzel et al., 2005 (58)</td>
<td>Rat</td>
<td>2 nM, 2.1 fold</td>
</tr>
<tr>
<td></td>
<td>Baar et al., 2005 (5)</td>
<td>Rat</td>
<td>ND, 2.5 fold</td>
</tr>
<tr>
<td></td>
<td>Naito et al., 2006 (60)</td>
<td>Rat</td>
<td>3 nM, 1.5-fold</td>
</tr>
<tr>
<td></td>
<td>Fink et al., 2000 (26)</td>
<td>Chicken</td>
<td>ND, 1.48-fold</td>
</tr>
<tr>
<td><strong>Calcium</strong></td>
<td>Zimmermann et al., 2000 (108)</td>
<td>Rat</td>
<td>0.24 mM, 5-fold</td>
</tr>
<tr>
<td></td>
<td>Fink et al., 2000 (26)</td>
<td>Rat</td>
<td>0.4 mM, 1.8-fold</td>
</tr>
<tr>
<td></td>
<td>Baar et al., 2005 (5)</td>
<td>Rat</td>
<td>5 mM</td>
</tr>
<tr>
<td></td>
<td>Zimmermann et al., 2006 (109)</td>
<td>Rat</td>
<td>0.4 mM, 1.5-fold</td>
</tr>
<tr>
<td></td>
<td>Fink et al., 2000 (26)</td>
<td>Chicken</td>
<td>3.7 mM, 2.2-fold</td>
</tr>
<tr>
<td></td>
<td>Eschenhagen et al., 1997 (22)</td>
<td>Chicken</td>
<td>4 mM, 3-fold</td>
</tr>
<tr>
<td></td>
<td>Schauf et al., 2011 (79)</td>
<td>Human</td>
<td>0.8 mM, 7-fold</td>
</tr>
<tr>
<td><strong>FDAR</strong></td>
<td>de Lange et al., 2011 (16)</td>
<td>Mouse</td>
<td>No change</td>
</tr>
<tr>
<td><strong>Force-frequency relationship</strong></td>
<td>Zimmermann et al., 2000 (108)</td>
<td>Rat</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Pong et al., 2011 (68)</td>
<td>Rat</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Eschenhagen et al., 1997 (22)</td>
<td>Chicken</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>de Lange et al., 2011 (16)</td>
<td>Mouse</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Lai et al., 2011 (50)</td>
<td>Mouse</td>
<td>Negative</td>
</tr>
</tbody>
</table>

FDAR, frequency-dependent acceleration of relaxation; ND, no data.
fers between embryonic/fetal and adult hearts with the sarcoplasmic reticulum becoming increasingly dominant with maturation (88). This may also affect the sensitivity of intact preparations to external Ca$^{2+}$. To which extent these factors account for the unusual Ca$^{2+}$ sensitivity of engineered cardiac constructs has not been addressed so far.

Several reports describe the existence of a sympathetic and parasympathetic receptor system on cardiac constructs (26, 60, 110) by demonstrating positive inotropic and chronotropic effects of isoprenaline and reversion by carbachol. The calculated half-maximal effective concentration for isoprenaline in these experiments was shown to be in the lower nanomolar range, which is ~10-fold less than those reported from studies in neonatal rat hearts and adult rat papillary muscle (~50 nM; (52, 91)). The relative isoprenaline-induced increase in force appears smaller in engineered constructs than in adult heart preparations (~2-fold vs. 4–5-fold, Table 1), which may again indicate immaturity. But it also relates to the increased sensitivity to external Ca$^{2+}$. For example, isoprenaline has no inotropic effect at all at physiological Ca$^{2+}$ concentrations of 1.8 mM, because contractile force is already maximal and cannot be further enhanced.

**Action potential and ion currents.** Action potentials of cardiomyocytes are driven by a unique set of Na$^+$, Ca$^{2+}$, and K$^+$ channels. Cardiac ion channel equipment and action potential characteristics differ considerably between laboratory animals (particularly mice and rats) and humans, developmental stages, cardiac regions, and disease states (90). A typical example is the transient outward repolarizing K$^+$ current, which is dominant in mice and rats and small in humans and guinea pig, strong in subepicardial and small in subendocardial layers and downregulated in failing human hearts (99). The advent of cardiac tissue engineering from human pluripotent cells is raising the question as to whether these cells/engineered tissues are electrophysiologically immature or not. Rather they are electrophysiologically immature cells resembling cardiomyocytes of 16-wk-old embryos (56). This immaturity is reflected by low maximal diastolic potential and K$^+$ currents and increased upstroke velocities (79, 103–104). Evidence suggests that prolonged culturing (27, 54), addition of nonmyocytes (45), and the 3-D format in EHT promote some aspects of maturation. Zhu et al. (104) demonstrated that the differentiation in the presence of neuregulin increased the percentage of mature cardiomyocytes with slower firing rate, increased upstroke velocities, and more negative maximum diastolic potential. Continuous electrical pacing improved neonatal rat cardiac constructs by transcriptional, histological, and contractile means (72) and may be a way to improve cardiomyocytes maturation in human constructs as well. Despite the immaturity of human cardiac myocytes from pluripotent stem cells, several studies showed effects of indicator drugs on single cardiomyocytes or engineered tissues (10, 31–32, 79). This opens the realistic perspective for their use in drug development and toxicology applications.

In summary, engineered cardiac constructs have gained increasing importance as advanced experimental test beds and as material for cardiac repair. Since their first description 15 years ago, four major developments toward applications have occurred. First, addition of mechanical loading and electrical stimulation, higher O$_2$-content, various medium supplements and/or miniaturization have led to improved 3-D cardiac tissue structure, maturation of myocytes, reduced extracellular matrix content, and development of near-physiological contractile force. Second, automation and standardization helped to set up an assay format suitable for medium throughput screening. Third, larger constructs composed of many small EHTs or stacked cell sheet tissues have been tested for cardiac repair and are associated with functional improvements in rats. Whether these approaches can be simply transferred to larger animals or the human patients remains to be tested. Theoretical arguments strongly suggest the necessity for preimplantation vascularization. Fourth, an unrestricted human cardiac myocyte cell source from hESCs or hiPS cells is available and represents a major breakthrough. Whereas drug testing with human engineered cardiac constructs is already reality, cardiac regeneration with such constructs remains a fascinating yet open perspective.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

T.E. conception and design of research; T.E. and A.H. drafted manuscript; T.E., A.E., I.V., and A.H. edited and revised manuscript; T.E., A.E., I.V., and A.H. approved final version of manuscript; A.E. performed experiments; A.E. and I.V. prepared figures.

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