This is a Review in a thematic series on **Repairing the Heart: Thinking Outside the Stem Cell Box**, which includes the following articles:

Patching the Heart: Cardiac Repair From Within and Outside [*Circ Res*. 2013;113:922–932]
Protein Engineering for Cardiovascular Therapeutics: Untapped Potential for Cardiac Repair [*Circ Res*. 2013;113:933–943]
Cardiac Tissue Engineering: State of the Art
3D Engineering in the Heart Chamber

Jeffrey Robbins, Editor

Cardiac Tissue Engineering
State of the Art

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Abstract: **The engineering of 3-dimensional (3D) heart muscles has undergone exciting progress for the past decade. Profound advances in human stem cell biology and technology, tissue engineering and material sciences, as well as prevascularization and in vitro assay technologies make the first clinical application of engineered cardiac tissues a realistic option and predict that cardiac tissue engineering techniques will find widespread use in the preclinical research and drug development in the near future. Tasks that need to be solved for this purpose include standardization of human myocyte production protocols, establishment of simple methods for the in vitro vascularization of 3D constructs and better maturation of myocytes, and, finally, thorough definition of the predictive value of these methods for preclinical safety pharmacology. The present article gives an overview of the present state of the art, bottlenecks, and perspectives of cardiac tissue engineering for cardiac repair and in vitro testing. (*Circ Res*. 2014;114:354-367.)**

Key Words: drug toxicity ■ guided tissue regeneration ■ heart disease ■ myocardium ■ organ culture techniques ■ organoids ■ pluripotent stem cells

The engineering of 3-dimensional (3D) cardiac tissue has been driven by 3 motives: to produce in vitro tissue surrogates for cardiac repair; to advance in vitro models of heart function; and by mere fascination of observing a heart muscle beating in the dish. Today, ≈20 years after the first creation of engineered heart tissue (EHT) from embryonic chicken cardiac myocytes,[1] >400 papers per year are published under the key words cardiac tissue engineering, a value matching that of hypertrophic cardiomyopathy (HCM). This may be surprising given that cardiac tissue engineering has not entered the clinical arena yet or has found wider application in preclinical drug development. However, with the enormous progress in stem cell biology and the widespread availability of human cardiac myocytes from pluripotent stem cells (PSCs), tissue engineering techniques are needed to reveal the full potential of these cells for drug screening and patient-specific disease modeling. Furthermore, the limited success of recent cardiac cell therapy studies calls for improved cell delivery methods that may be realized by tissue engineering. Active research and development programs are currently directed toward these aims, the first patient has been treated with a stacked (noncardiac) cell sheet patch,[2] and some of the 3D assays are ready to be applied in automated drug testing. This article gives an overview of the methods to create 3D heart tissues in vitro and the 2 major applications with their respective achievements (eg, in vitro vascularization), open questions, and perspectives. The history of cardiac tissue engineering has been reviewed in detail previously.[3]

Methods for Engineering Heart Tissue

Principles: Hydrogels, Matrices, Cell Sheets

Making 3D heart tissues is principally an easy task because (immature) cardiac cells not only beat spontaneously but also have an intrinsic capacity to form 3D functional syncytia. In the late 1950s, Moscona[4] showed that simple

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Nonstandard Abbreviations and Acronyms	
cMyBP-c	cardiac myosin-binding protein-C
ECM	extracellular matrix
EHT	engineered heart tissue
hESC	human embryonic stem cells
HCM	hypertrophic cardiomyopathy
hiPSC	human induced pluripotent stem cells
PKCα	protein kinase C (isoform α)

gyratory shaking of embryonic chicken cardiac myocytes in an Erlenmeyer flask induced the formation of spontaneously beating spheroids with improved functionality when compared with standard 2D-cultured myocytes. This self-assembly is still used in variations to generate cardiac microspheres.[5] Current cardiac tissue engineering methods embark on this endogenous capacity and use engineering techniques to make 3D constructs of the desired size, geometry, and orientation (Table).

Hydrogel Technique

The hydrogel method has been pioneered in the 1980s as an advanced culture method for fibroblasts and skeletal muscle cells[6] and gave rise to the first successful cardiac tissue.[7] It needs 3 factors: solutions of gelling natural products, such as collagen I, matrigel, fibrin, or mixtures of them; casting molds; and anchoring constructs. The hydrogel entraps cells in a 3D space during gelling, the mold gives the 3D form, and the anchors allow the growing tissue to fix and to develop mechanical tension between ≥2 anchor points. Important aspects of this technique are that the naturally occurring hydrogels stimulate cells to spread and form intercellular connections and that the cells compress the gel, reduce the water content, and thereby shrink it in size several-fold. Anchoring exposes the growing tissue to a continuous mechanical strain and induces orientation of cells parallel to the force lines. Mechanical stability is lower than in scaffold-based techniques, but this methodology is easily miniaturized and automated and allows standardized force measurements. Therefore, it is well-suited for in vitro testing.

Prefabricated Matrices

A second tissue engineering approach has been developed from the engineering side and seeds prefabricated porous solid matrices with cardiac cells, similar to what has been performed in the bone and cartilage tissue engineering field. Various materials have been tested, including alginate,[8] collagen,[9] and gelatin sponges,[10] polyglycolic acid,[11] poly-L-lactic acid/polyglycolic acid composites,[12] and poly(glycerol sebacate).[13] An obvious advantage when compared with other techniques is the ease to engineer any desired 3D form and manipulate constructs in culture. Integrating small electrically conducting chemically inert gold nanofibers into an alginate construct improved tissue formation.[14] The most advanced 3D constructs in terms of tissue structure and function were produced with collagen sponges and chronic electric pacing, likely because the natural environment provides a better environment for the cells than the artificial polymers.[9]

Decellularized Heart Tissue

Exploiting nature's principles directly is the driving force behind widely publicized attempts to make an artificial heart by decellularizing whole hearts and repopulating them with cardiac cells.[15] Decellularization is performed by extended Langendorff perfusion with SDS and Triton X-100, a procedure that almost completely eradicates cells from the tissue but leaves connective tissue architecture of blood vessels intact. This allows perfusing of the remaining matrix but makes repopulation with cardiac cells difficult.

Cell Sheets

Cardiac myocytes, cultured on standard plastic dishes for extended periods, tend to detach from the substrate as a more or less intact monolayer. Shimizu et al[16] exploited this principle by developing temperature-sensitive coating materials that allow cell monolayers to detach as intact monolayers by leaving the culture dish at room temperature. Stacking of several cell sheets generates 3D tissues that beat and develop force. Similar approaches have been developed early for the engineering of blood vessels.[17] This scaffold-free technology is versatile and well-suited for transplantation. Accordingly, there are active developments to automatize this technology for cardiac repair.[18] Detachment from culture surfaces has also been used to generate little cylindrical muscles attached to steel pins that can then be subjected to force measurements.[19]

Lessons

Twenty years of cardiac tissue engineering have taught a number of lessons regarding how to optimize cardiac tissue quality. Most of this work has been performed with neonatal rat heart cells, which therefore are referred to in this paragraph. 3D constructs from human PSCs (hPSCs) are discussed later.

Strain

Mechanical strain is crucial for cardiac myocytes to align and to maturate as shown in numerous studies with hydrogel-based EHTs. The simplest form of strain is static tension because it develops in EHTs fixed between 2 glass rods,[7] in a ring around a central rod,[20] in tubular casting molds around a mandrel[21] or as a strip between 2 steel needles,[19] or other rigid anchoring devices.[22] Improved cardiac tissue structure and higher force development have been observed in EHTs that were exposed to phasic 10% to 15% stretch by a motorized stretching device[23] or in EHTs cultured in a manner that they can beat and perform work against elastic anchoring points.[24,25] In conclusion, methods that allow EHTs to perform auxotonic contractions are best suited, probably because they imitate the normal conditions of the heart contracting against the hydrostatic pressure of the circulation.

Electric Stimulation

Heart tissue continuously beats under the control of pacemaker cells in the sinoatrial node. Engineered cardiac constructs also beat spontaneously but at varying rates and with some irregularity over time. Experiments with a composite method of seeding collagen sponges with cardiac cells in Matrigel have shown that pacing for 8 days improved cell orientation, tissue structure, and function.[9,26] This was reflected by a more physiological volume fraction ratio of myofilaments:mitochondria:nuclei

Table. Different Approaches in Cardiac Tissue Engineering and Their Application in Cardiac Regeneration and In Vitro Testing

Approaches		First Introduction	Animal Cell Type	Human Cell Type	Transplantation in	Physiological/Pharmacological Testing	Drug Testing	Disease Modeling/ Functional Genomics	References
Cells immersed in hydrogel									
Collagen	Biconcaval lattices	1997	Embryonic chicken	…	…	Frank Starling, force–frequency, $Ca,^{2+}$ Iso, CCh	…	…	7
Collagen+matrigel	Rings	2000	Neonatal rat Mouse parthenogenetic stem cells[68]	hiPSC[92]	Immune-suppressed rat heart,[52] infarcted rat heart,[25] mouse heart[68]	Frank Starling, force–frequency, $Ca,^{2+}$ Iso, CCh[76]	Statins[93]	shRNA KD of PKCα[78]	20,22,23,25, 31,33,37,52, 68,76,78,87, 92,93
	Flexcell tissue train	2011	Neonatal mouse[22,87]	hESC+hiPSC[33]	Nude rat[33]	Frank–Starling, force–frequency, Fura-2 Ca^{2+} transients[22]	…	cMyBP-C KO mouse[87]	
	Titanium rods	2011	Neonatal rat cardiomyocytes, Mouse iPSC[31]	Human ESC and iPSC[31]	…	Frank–Starling, Iso, CCh[31]	…	…	
Fibrin	Self-organizing planar networks	2009	Neonatal rat,[51] mouse ESC, and CV progenitor cells[77]	…	Rat omentum	Frank–Starling, force–frequency	…	…	51,77
	Tubular molds	2009	Neonatal rat	…	…	…	…	…	21
	Elastic silicone posts	2010	Neonatal rat	hESC[34]	…	Frank–Starling, force–frequency, $Ca,^{2+}$ Iso, CCh[24]	Proarrhythmic compounds[24,34]	Afterload-induced hypertrophy,[72] hypertr.+endothelin receptor antagonists[72]	24,34,72
Fibrin+collagen	Upright silicone posts	2012	Neonatal rat	…	…	Iso, Digoxin, fluo-4 Ca^{2+} transients	…	…	73
Cell-seeded bioresor bable scaffolds									
Collagen sponges	Gelfoam disks	1999	Fetal rat	…	Cryo-injured rat heart	…	…	…	10
	Ultrafoam disks+matrigel	2003	Neonatal rat	…	…	Excitation threshold	…	Chronic pacing	9
PGA	Fibrous meshes	1999	Neonatal rat	…	…	…	…	…	11
Alginate	Spheric disks	2000	Fetal rat	…	Infarcted rat heart	…	…	…	8
PLLA/PGA composite	Squared sponges	2010	…	hESC±HUVEC and MEF	Immune-suppressed rat heart	…	…	…	12
PGS	Perfusion seeding of disks	2010	Neonatal rat	…	Infarcted rat heart	…	…	…	13
Cell sheets									
Thermosensitive coating of culture dishes, stapling of sheets		2002	Neonatal rat,[16] mouse ESC[94]	hiPSC[64]	Subcutaneous tissue of nude rats, heart,[16] in vitro perfused collagen gel,[59] infarcted porcine heart[64]	…	…	…	16,22,59,64, 94
Spontaneous detachment of monolayer+fixation on steel pins		2005	Neonatal rat	…	…	…	$Ca,^{2+}$ epinephrine	…	19

(*Continued*)

Table. Continued

Approaches	First Introduction	Animal Cell Type	Human Cell Type	Transplantation	Physiological/Pharmacological Testing	Drug Testing	Disease Modeling/ Functional Genomics	References
Scaffold-free self-organization								
Gyratory shaking in Erlenmeyer	1959	Embryonic chicken	—	—	—	—	—	4
Hanging drop or agarose molds	2004	Neonatal rat and mouse	—	Chorioallantoic membrane chicken, adult rat pericardium	—	—	—	5,95
Gyratory shaking in 6-well dish	2009	—	hESC±HUVEC, MEF, or dermal FB	Nude rat	—	—	—	32

CCh indicates carbachol; cMyBP-C, cardiac myosin-binding protein-C; CV, cardiovascular; ESC, embryonic stem cells; FB, fibroblast; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; HUVEC, human umbilical vein endothelial cell; iPSC, induced pluripotent stem cells; Iso, isoprenaline, KD of PKCα, knockdown of protein kinase C (isoform α); MEF, mouse embryonic fibroblast; PGA, polyglycolic acid, PLLA, poly-L-lactic acid; and PGS, poly (glycerol sebacetate).

from 10%:8%:27% in unpaced constructs to 32%:10%:15% in paced constructs (compared with 40%:20%:5% in adult heart).

Nonmyocytes

Heart tissue is composed of myocytes (20%–30% in human heart,[27] 50% in mouse heart,[28] and 30% in rat heart)[28] and nonmyocytes, such as fibroblasts and endothelial and smooth muscle cells. Similarly, collagen I–based EHTs contain non myocytes plus macrophages at high density, intricately intermingled with cardiac myocytes. Numerous lines of evidence suggest that nonmyocytes support the growth of myocytes in culture and are important for generating engineered constructs. First, EHTs made from an unpurified, native heart cell mix developed 3× higher forces than those made from purified cardiac myocytes.[29] Second, poly(glycerol sebacate) constructs seeded consecutively with cardiac fibroblasts and myocytes developed much better tissue structure and revealed lower activation threshold than those cultured with pure myocyte.[30] Third, pure, genetically selected cardiac myocytes from mouse and hPSC did not form 3D engineered tissue but required the addition of fibroblasts.[31] Finally, the addition of endothelial and stromal cells to human embryonic stem cell (hESC)–derived cardiac myocytes consistently improved cardiac tissue structure and function.[12,32,33]

If nonmyocytes are essential for tissue building, then it is surprising that relatively well-functioning EHTs can be generated from hPSC-derived cardiac myocytes without addition of other cells.[34] This study used unpurified cells derived from a growth factor–based protocol that yielded ≈40% to 60% cardiac myocytes. The rest of the cells were positive for vimentin, a nonspecific marker of fibroblasts. Therefore, it is most likely, but not proven, that these cells helped to make the tissue. Whether different types of fibroblasts or other cell types improve tissue quality in a differential manner remains to be studied. It is also not clear whether paracrine, direct cell–cell interactions, or other factors underlie the beneficial effect of nonmyocytes. Fibroblast-produced extracellular matrix (ECM) stimulated the growth of ventricular rat cardiac myocytes even when fibroblasts were removed, arguing for an ECM-mediated mechanism.[35]

Spontaneous Vascularization

3D cardiac constructs made from primary cardiac myocytes develop a primitive vascular network without[20,29] or with the addition of endothelial cells.[32,36] Importantly, the cellular networks clearly form lumina. At present it is not clear whether, in the absence of perfusion, these structures serve more than a paracrine function; consistently, they participate in the process of in vivo vascularization.

Maturation

It is one of the central promises of the field that cardiac myocytes in a 3D cardiac tissue acquire a more mature phenotype than those cultured on rigid plastic dishes. Arguments for a higher degree of cardiac differentiation and maturation in 3D models, such as EHTs, made from neonatal rat cardiac cells are strictly the following: longitudinal orientation and alignment; high degree of sarcomere structure; relatively normal ratio of sarcomeres; mitochondria and nuclei; formation of M-bands, rod-shaped cardiac myocytes; high degree of binucleation;

normal diastolic resting potential of approximately −90 mV; relatively normal action potential shape; high abundance of sarcomeric and sarcoplasmic proteins; and qualitatively normal physiological and pharmacological responses—such as Frank–Starling behavior, force–frequency relationship, and reaction to calcium, isoprenaline, and carbachol.[7,20,22,24,37] Similar features were observed in stacked cell sheets[16] and myocyte-populated collagen sponges after electric stimulation.[9] However, it is also fair to state that just putting cells in a 3D environment does not make them better. Importantly, even with the best 3D model, cardiac myocytes in culture do not acquire a fully mature phenotype yet. For example, sarcomere and mitochondrial volume fractions remain less than in the adult heart (ie, myocytes are not so densely packed with contractile machinery as in the adult state,[20] cells have an unusual length/width ratio of 12:1 [adult 5:1], the ratio of the adult α-myosin heavy chain isoform to the fetal β-isoform was 7 in 12-day-old EHTs when compared with >100 in adult rat heart).[37] EHTs beat spontaneously (whereas adult ventricular tissue does not), and contractile forces remain less than those of isolated heart preparations if referred to the cross-sectional area.

Further improving cardiac myocyte maturation in 3D constructs is one of the central goals. Factors that will likely have an effect on the final maturity of cardiac tissue in vitro are age (time of culture), culture media composition, and optimized oxygen and nutrient supply (bioreactors and perfusion). It is likely that full maturation can only be reached if all factors are being optimized and combined with optimal, auxotonic conditions of contraction. A factor that has received surprisingly little attention in this respect is energy metabolism, despite the fact that the heart is metabolically extremely active, and a dramatic metabolic switch occurs during cardiac development and postnatal growth. These metabolic changes have important effects on the ability of the cardiomyocyte to proliferate and, later in development, terminally differentiate. The adult heart is fueled mainly by oxidative metabolism of fatty acids (and only a minor fraction of carbohydrates), the fetal heart, in contrast, mainly depends on glycolysis. Standard culture media are glucose-based and do not contain fatty acids. Fetal calf or horse serum, generally added to cardiac myocyte cultures at 5% to 10%, contain protein-bound fatty acids, but it is not known how much of this is used for metabolism and to which extent the lipid fraction adds to the permissive effect of serum in cardiac cell cultures.

Hypoxia Resistance

Neonatal rats and rat heart cells have a surprising degree of resistance against hypoxia. Newborn rats can be put in pure nitrogen for 30 minutes and longer without apparent damage. Day 1 rats even survive 30 minutes of pure CO_2 (ie, hypoxia and hypercapnia), whereas 10-day-old rats die after 5 minutes.[38] This likely reflects the relatively immature state of the litter in this species (eg, compared with piglets), relates to glycolytic metabolism, and represents an advantage at birth. Resistance to hypoxia certainly not only is an advantage for cardiac tissue engineering but also confounds conclusions as to the oxygen supply in tissues. For example, the fact that cell distribution is relatively homogeneous in EHTs does not necessarily prove oxygen supply. In fact, experiments with oxygen sensor probes showed concentrations of 2% and less inside of large fibrin-EHTs.[39]

Heart Tissue for Cardiac Regeneration

Different Approaches and Promises

In a traditional sense, tissue engineering aims at providing living, force-producing heart muscle tissue that can be transplanted on injured or malformed hearts and can restore normal function. This approach thus targets the primary defect, for example after myocardial infarction, and promises a causal therapy. In this sense, it is similar to cell therapy with a number of theoretical advantages. The notoriously low cell-retention rate seen after cell injection[40] is expected to be much higher with the fixation of intact patches, but this has not been proven yet. This is relevant with regard to efficacy and also safety, given that potentially tumorigenic cells, such as ESCs, were shown to be disseminated into the body.[40] Cell selection has been performed in vitro, reducing the side effects of massive cell death in situ. Finally, tissues can be better quality-controlled before implantation, and living muscle tissue should provide direct and definite support of the injured heart, whereas mechanisms of cell therapy seem largely independent of the addition of new contractile mass. Importantly, the potential advantages of the tissue engineering approach have not been proven yet in a direct and systematic head-to-head comparison with cell therapy, and logistics are certainly much more complex.

Whereas the tissue patch approach seems already ambitious with numerous biological, logistic, and regulatory hurdles, the field also works on the generation of biological assist devices[41–43] or even entire artificial hearts on the basis of decellularized hearts.[15] On the other side of the spectrum, tissue engineering in situ follows the idea that injection of biomimetic scaffolds into an injured heart stimulates endogenous repair processes. The concept is appealing in its simplicity. Accordingly, first clinical studies with alginate injections after myocardial infarction are underway (clinicaltrials.gov NCT01226563, NCT01311791). A recent study of infarcted pigs reported exciting therapeutic efficacy of injections of soluble ECM made from pig hearts.[44] It is not clear at this point how the reported formation of new myocardium is compatible with the extremely low capacity of the adult mammalian heart to regenerate cardiac myocytes.[45] Most studies to date concluded that the reported beneficial effects of injecting alginate,[46] ECM,[47] or nanofibers[48] into infarcted hearts are likely independent of the formation of new heart muscle and potentially because of improved angiogenesis or mechanical stabilization of the ventricular wall. Similar mechanisms have been suggested to account for the beneficial effects of cell therapy.[49] It needs to be seen whether these effects are sufficient to provide a long-term restoration of function.

To improve the results of cell therapy, others have injected cells in hydrogel solutions or as microspheres into the heart, hypothesizing that the hydrogel environment will augment retention of cells, survival, and growth.[50] Whereas the washout rate is certainly lower with cells trapped in a gel, it is less clear how this approach shall reduce one of the causal factors of the high cell death rate after cell injection (ie, the mechanical stress imposed on the cells by injecting them into a tight and relatively rigid tissue like the beating heart).

To circumvent both the problem of low cell retention and high cell death, others have seeded (nonmyocyte) cells (eg, mesenchymal stem cells and skeletal myoblasts) onto degradable biopolymers and transplanted them onto injured hearts. An elaborated approach was developed by Godier-Furnémont et al.[47] Scaffolds were made from decellularized human hearts, seeded with mesenchymal stem cell in a fibrin hydrogel, and implanted onto infarcted nude rat hearts. This promoted an arteriogenic response and improved functional recovery, particularly when mesenchymal stem cells had been in vitro preconditioned by transforming growth factor-β before cell seeding.

Heart Muscle Patches: State of the Art

Early studies have shown anecdotally that patches of cardiac myocyte-seeded scaffolds can be fixed on the living heart with cells surviving on the heart for limited periods.[8,10] Most systematic work has been performed with hydrogel-based EHTs,[25,33,51,52] stacked cell sheets,[36,53] and scaffold-free cell aggregates.[32] This work has been encouraging and has taught a number of lessons.

Survival Rate

Implanted 3D heart muscle patches survive on host hearts for extended periods. This is not self-evident given that metabolically active, often several 100-μm-thick tissues were implanted on the epithelialized surface of host hearts in the absence of immediate perfusion. Several reasons may account for it. First, cardiac myocytes from neonatal rat heart (and likely also ESCs) are, in stark contrast to adult myocytes, hypoxia-resistant and can live on anaerobic glycolysis at least for some time (see Hypoxia Resistance section of this article). Second, hydrogel-based or scaffold-based 3D constructs, in contrast to adult heart tissue, are relatively loosely packed with cells. Collagen EHTs exhibit an interbranching 3D network of elongated, relatively thin individual cells (5–7 μm in width).[37] This certainly facilitates diffusion. Third, vascularization from underlying blood vessels occurs relatively quickly. Yet, it is also clear that the period before true vascularization is critical and causes cell death, and the extent of which is difficult to quantify and has not been studied in detail. It certainly depends on the tissue density, as stated previously, but the cell sheet transplantation experiments of Shimizu et al[54] show that widespread necrosis occurs if >3 sheets (80-μm compact cardiac myocytes) are implanted in 1 step.

Vascularization In Vivo

Blood vessel ingrowth and perfusion are undoubtedly a prerequisite for long-term survival and function of a transplanted graft. It has been observed to occur quickly, both in subcutaneous[54] and in cardiac implantation sites.[12,25,32,52] In a systematic series of experiments in subcutaneous dorsal pockets of nude rats, Shimizu et al[54] showed that 1-day to 2-day intervals were sufficient between repeated implantations of 3-layer cell sheets to allow the building of thick cardiac tissue, whereas the single-step implantation of sheets made from >3 layers resulted in necrosis. Whereas this approach will be difficult to translate into clinical practice, it elegantly shows that vascularization is required to make thicker tissues and that it occurs fast. Whether the kinetics of vessel ingrowth is similar on the surface of the heart is difficult to study for practical reasons and remains unknown at present.

Role of Endogenous Vessel Structures

As outlined an early, initially surprising finding is that the endothelial and other nonmyocyte cells that are contained in the primary heart cell mix or that are added exogenously spontaneously form a primitive tubular network in 3D constructs. There are indicators that these structures participate in the process of vascularization after transplantation.[52] First, 4′,6-diamidin-2-phenylindol labeling of EHTs before implantation demonstrated that 4 weeks after implantation on infarcted rat hearts, blood-perfused vessels in the graft were often 4′,6-diamidin-2-phenylindol-positive (Figure 1A).[25] Second, cell sheets made from neonatal rat myocytes and green fluorescent protein-positive endothelial cells were quickly vascularized after implantation on hearts and blood perfused with partially green fluorescent protein-positive vessels (Figure 1B).[36] Similar findings were made with hESC-derived grafts supplemented with human umbilical vein endothelial cells and mouse embryonic or human dermal fibroblasts.[32,33] Finally, transplantation results were better in grafts containing preformed vascular structures than those made from pure hPSC-derived cardiac myocytes.[12,33,55] In conclusion, endothelial and stromal cells are required in addition to cardiac myocytes to make optimal cardiac muscle tissues for transplantation.

Vascularization and Perfusion Before Transplantation

Tissues with preformed vascular structure are often termed vascularized cardiac grafts. This terminology is partially misleading because they neither are perfused in vitro nor can be directly connected to the host circulation, both requisites for the production of sufficiently thick and strong grafts. Much recent progress has been made toward this goal. Tee et al[56] isolated arteriovenous loops of rat epigastric arteries, cast neonatal rat cardiac cells in Matrigel in silicone chambers around the loops, closed the chamber, and let the cardiac tissue grow for several weeks. This resulted in the formation of a well-vascularized cardiac tissue with a defined arterial and venous pedicle that, when connected to blood vessels of the neck, stably perfused the tissue fixed to the heart (Figure 1C–1E). Sekine et al[57] transferred this approach into an in vitro setting, integrated an isolated piece of perfused rat grown tissue into a chamber, connected the artery and vein to a medium perfusion system, and implanted cardiac cell sheets onto the vascularized surface. Blood vessels grew into the sheets and allowed repeated implantations and the generation of thicker, fully vascularized grafts that survived after connection to a recipient's circulation and transplantation in the neck region of rats (Figure 1G). Radisic et al[58] developed an elegant in vitro approach in which 2 blood vessel explants are embedded on 2 opposing sides of a collagen–chitosan hydrogel-releasing thymosin β4, layered on micropatterned silicone surfaces. This stimulated the longitudinally directed outgrowth of capillaries from the vessels, which after ≈2 weeks started to connect the 2 explant vessels, allowing directed perfusion. In a second step, cardiac myocytes were then cultured on top of the vascularized hydrogel- bed (Figure 2A–2E).[58] Whereas in the latter approach endothelial cells from blood vessels explants are used to form capillaries that support cardiac myocytes, endothelial cells contained in cardiac cell sheets were also shown to be able to connect to noncellularized microchannels in collagen

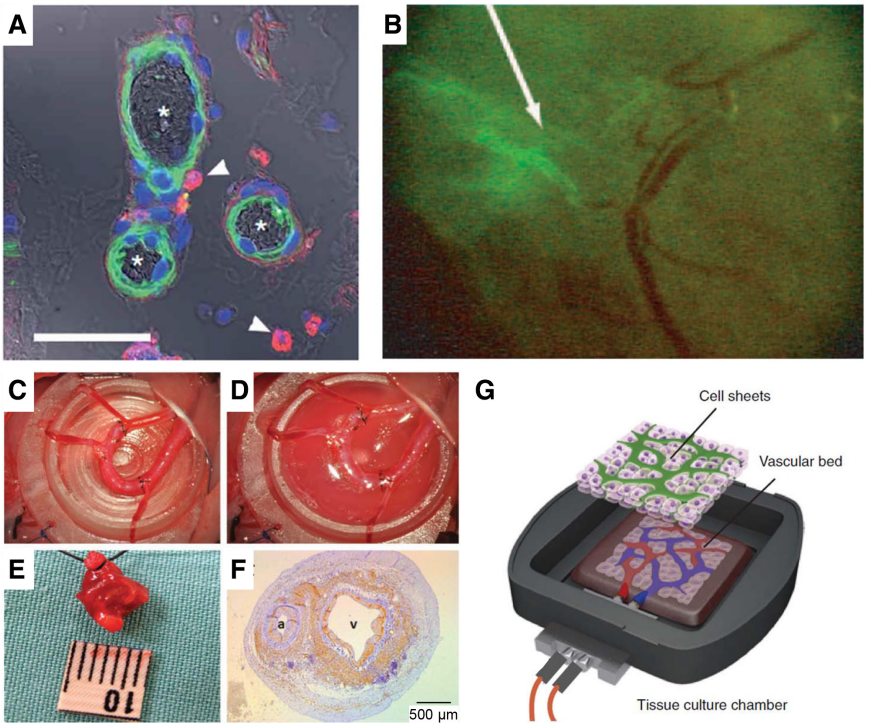

Figure 1. Vascularization of 3-dimensonal (3D) engineered tissues. **A**, Early evidence for the contribution of preexisting vascular structures in 3D cardiac grafts. 4′,6-diamidin-2-phenylindol (DAPI)-labeled engineered heart tissues were transplanted onto infarcted rat hearts and analyzed 4 weeks later. Blood-perfused vessels in the graft were DAPI$^+$ (actin, green; nuclei [DAPI], blue; macrophages [CD163], red/arrows; erythrocytes, asterisks). Scale bar, 50 μm. Reprinted from Zimmermann et al with permission of the publisher.[25] **B**, Evidence that invading blood vessels of the host connect to preexisting vascular structures in grafts. Three-layer cardiac cell sheets cocultured with green fluorescent protein (GFP)-positive endothelial cells were transplanted onto infarcted rat hearts. Note that a GFP$^-$ blood vessel of the host heart connects to a GFP$^+$ capillary from the transplanted cardiac graft. Reprinted from Sekine et al with permission of the publisher.[36] **C**–**F**, In vivo bioreactor approach in syngeneic rats. **C**, An arteriovenous loop fashioned in the groin of inbred Sprague–Dawley rats was placed into the base of a polycarbonate chamber. **D**, Neonatal cardiomyocytes suspended in Matrigel were implanted into the chamber. **E**, Gross appearance of the engineered myocardial flap at 8 weeks. **F**, Cross–section of the 8-week myocardial flap showing the patent femoral artery (a) and vein (v) with cardiac muscle–like tissue stained with desmin immunohistochemistry (scale bar, 500 μm). **C**–**F**, Reprinted from Tee et al with permission of the publisher.[56] **G**, In vitro native vascular bed approach. Three-layer cardiac cell sheets cocultured with endothelial cells were placed on top of a resected vascular bed positioned in a bioreactor. The cocultured endothelial cells formed new blood vessels and connected with the blood vessels that originated from the vascular bed. The in vitro vascularized cardiac tissues beat and could be anastomosed to the recipient's circulation. Reprinted from Sekine et al with permission of the publisher.[57]

gels (Figure 2F).[59] Similarly, microchannels in EHTs generated by enzymatically dissolving alginate-fibers embedded in the EHT reconstitution mix during casting were populated by endothelial cells. Prolonged perfusion improved cardiac tissue structure and density (Figure 2G).[39] Many of these approaches still allow only low perfusion rates because of the lack of a real blood vessels structure. However, the way to produce clinically relevant thick grafts ready to be connected to the coronary circulation seems to be apparent, and this constitutes major progress in cardiac tissue engineering.

Electric Coupling

Grafts need to be electrically coupled to the host heart to support its function. Because coupling is not expected to occur in the infarcted zone of a recipient's heart, most researchers take care to implant tissues/cell sheets in a way that the edges are in contact with healthy myocardium. Full electric coupling between graft and host heart has been observed both with EHTs[25] and with cell sheets.[53] Still, coupling is not self-evident. Both the host heart's epicardium and an epithelial cell layer on the surface of grafts[52] separate myocytes from direct coupling. Attempts to remove the heart's surface layer lead to uncontrollable bleeding. Moreover, implantation consistently causes the formation of a layer of cell-free ECM between the host myocardium and the graft,[25,36,52,54] likely as part of a wound healing process and similar to what has been reported from cell injections.[60] Accordingly, direct histological proof of coupling is sparse. This can indicate that few cardiac myocyte–myocyte connections are sufficient to propagate either the electric signal or the enhanced conduction through the infarct zone might be occurring in host cells salvaged by paracrine benefits. An alternative explanation would be electrotonic coupling via cardiac fibroblasts that has recently been experimentally supported in other contexts.[61]

Therapeutic Effect

Repairing the defective heart by implantation of a patch and formation of new muscle tissue is the central promise of the field. A proof-of-concept study in >340 rats has shown that the implantation of multiloop EHTs 4 weeks after large myocardial infarctions improved parameters of diastolic and systolic function without normalizing them.[25] In this study, patches were made with 12.5 million cells (starting number), of which 4 million survive for 12 days in vitro.[37] An unknown fraction of these cells die after implantation, but the generation of new myocardium was observed with a mean thickness of 440 μm. Is this sufficient to account for the increase in function? The adult rat heart contains ≈30 million cardiac myocytes. If one estimates one-third of the muscle mass to be destroyed by a proximal left anterior descending artery ligation, then 10 million myocytes are lost and the addition of 2 million (assuming a survival rate of 50%) could give rise to the improvement in function by ≈20% to 30%. An important argument for this conclusion is that parallel implantation of nonmyocyte grafts did not have therapeutic effects in this study.[25] However, numerous studies with much smaller 3D constructs or simple injections of various nonmyocyte cell types have reported comparable therapeutic success in this or similar animal models.[36,49] This indicates that other mechanisms, such as improved

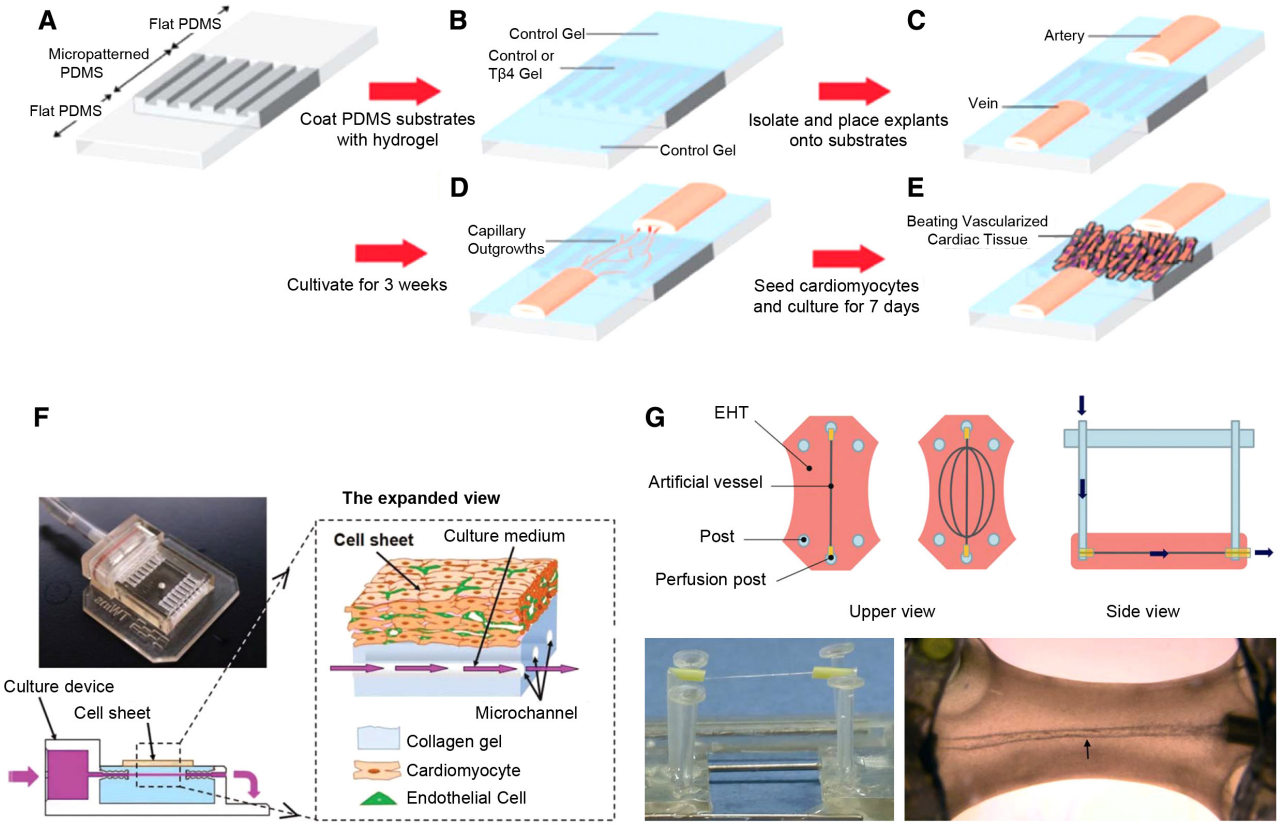

Figure 2. In vitro vascularization of 3-dimensional engineered tissues guided by micropatterns. **A–E**, Sequential generation of vascularized engineered tissue. **A**, Polydimethylsiloxane (PDMS) with microlanes was used as a substrate. **B**, After coating with collagen–chitosan hydrogel with or without angiogenic thymosin β4 (Tβ4) and (**C**) arteries and veins were isolated and placed on the 2 ends of the substrate. **D**, Three weeks later, capillary outgrowths connected and (**E**) cardiomyocytes were seeded onto the engineered vascular bed and cultured for additional 7 days to grow a beating, vascularized cardiac tissue. Reprinted from Chiu et al with permission of the publisher.[58] **F**, Perfusion bioreactor for cell sheet technology. Triple-layer cell sheets were incubated on a cell-free collagen gel base with perfused microchannels (Ø=300 μm). The culture medium could diffuse into the collagen gel and provide oxygen and nutrients to the cell sheet. Endothelial cells in the cell sheets migrated into the collagen gel and finally connected with the microchannels. Reprinted from Sakaguchi et al with permission of the publisher.[59] **G**, The alginate fiber vascularization technique applied to hydrogel-based engineered heart tissue (EHT). By dissolving alginate fibers with alginate lyase, microchannels were created and they endothelialized over time (here filled with air for better visualization). Reprinted from Vollert et al with permission of the publisher.[39]

angiogenesis, reduced fibrosis formation, paracrine effects, or mere mechanical stabilization, may also be involved.

Heart Muscle From hPSC

For many years, it was not clear how to transfer tissue engineering approaches that were all developed and tested in chickens or rodents to a human setting. With the exciting progress in stem cell technology, this has changed. EHTs, cell sheet, and scaffold-based 3D constructs have been made from hESC and human induced PSC (hiPSC).[33,34,55,62–64] Tissue structure and function are still not as good as that of rat 3D constructs, but cocultures[32,33] and mechanical and pacing conditioning,[26] as well as advanced growth factor protocols, will improve these parameters. Implantation experiments suggest that human 3D cardiac constructs behave similar or even better than their rat counterparts.

Bottlenecks

With the increasing efficiency of large-scale productions of cardiac myocytes from hPSC (eg, GE Healthcare, Cellular Dynamics International, Axiogenesis, and Cellartis/Cellectis providing cardiac myocytes on a commercial basis), the availability of cells for the generation of large tissue patches soon will not be limiting anymore. Still, significant hurdles exist regarding the first implantation of a true heart muscle patch in a patient.

Size

The dimensions of cardiac patches in width and length have no theoretical limit, and several means to enlarge 3D constructs on surfaces have been developed.[25,51] However, the maximal viable thickness in the absence of directed perfusion remains a critical but only partially solved issue. The cell sheet data suggest that 80 μm of compact muscle (ie, a structure in which 1 layer of myocytes directly follows the other) is a critical limit.[54] Similarly, compact muscle strands in EHTs rarely exceed a thickness of 100 μm, despite the fact that EHT thickness overall can be several millimeters. Thus, in vitro vascularization and perfusion are likely a mandatory requirement for the generation of tissues that, for realistic repair of a large infarction scar, need to be several millimeters in muscle thickness.

Immune Responses

Until now, all transplantation studies have been performed under immune suppression,[25] in syngeneic,[52] or in immunodeficient

animals.[32,36] Early data regarding immune privilege of ESC were not reproducible.[65] Autologous approaches based on hiPSC were initially considered the solution, but even iPSC derivatives can elicit immune responses in some studies,[66] whereas others found no signs of rejection.[67] However, the autologous approach takes a minimum of 6 to 9 months and may impose logistic issues that are difficult to solve. Each hiPSC line is, from a regulatory point of view, a new product and likely needs to undergo the entire battery of toxicology studies before the first application. It may make this approach prohibitively expensive and too time-consuming. An alternative is a well-characterized bank of hESC or hiPSC lines that offers human leukocyte antigen-matched products for most patients, who will then only require a minimal immune suppression. A fascinating possibility to reduce the number of possible combinations of surface antigens substantially while escaping the ethical dilemma of hESC is the use of parthenogenetic stem cells. Given the uniparental origin of these cells, the number of myosin heavy chain combinations is considerably reduced, suggesting that only a fraction of parthenogenetic stem cell would make a good bank. In mice, parthenogenetic stem cells, similar to mESCs, readily differentiated into cardiac myocytes that were used to make EHTs and transplanted heart patches.[68] Yet, similar to iPSC, reports about genomic instability of parthenogenetic stem cell warrant further research.[69]

Safety and Efficacy

The 2 central challenges of all types of therapy impose a particular problem to cardiac tissue engineering because other than drugs, testing in vitro (eg, receptor-binding assays) or in small animals is not possible or rather meaningless. Implanting human heart patches on mouse or rat hearts faces the problem of widely different cardiac physiologies, exemplified by 5-fold to 10-fold higher heart rates and substantially different types of action potentials. Thus, testing has to be performed in animals with a cardiac physiology closer to that of humans (eg, guinea-pig, rabbit, pigs, dogs, or nonhuman primates, the latter with considerable ethical and financial implications). Furthermore, the product (ie, the human heart patch) has to be tested as such and cannot be substituted with, for example, a pig heart patch. Thus, testing is xenogenic and has its own problems. Safety issues are of particular relevance in hiPSC-derived products because, in addition to the general risk of teratoma formation in PSC, chromosome instability and the introduction of mutations can create increased risk of malignant transformation. The Food and Drug Administration and European Medicines Agency have recently formulated guidelines addressing the special issues of cell therapy products (EMA/CAT/571134/2009), but the requirements are high. Geron Corporation had to submit a 22 000-page document to the Food and Drug Administration to perform the first hESC trial in patients with spinal cord injury.[70]

Perspectives

Despite these hurdles and foreseeable problems, including costs, the field is moving forward. Groups in the United States, Europe, and Japan run active programs to perform the first safety and efficacy trial in pigs and nonhuman primates. These studies will be closely monitored by industry. Geron stopped the first hESC therapy trial prematurely, not for safety or efficacy problems but for economic reasons. Despite this largely echoed decision, Advanced Cell Technology currently runs a hESC-based trial in macular degeneration, the results of which are eagerly awaited (NCT01345006).

Heart Tissue as an Advanced In Vitro Model

Promises

Experiments in cultured cardiac myocytes have taught a lot about molecular processes controlling the growth and proliferation of cardiac myocytes, but translating these findings to human cardiovascular disease is difficult. Reasons include the highly artificial culture conditions of isotropically oriented cells on rigid plastic surfaces, as well as species differences. The availability of human cardiac myocytes derived from hESC or hiPSC has principally solved the latter issue. However, tissue engineering techniques promise that cells studied in a 3D cardiac tissue context behave more similar to those in vivo but can be manipulated without the interference of systemic factors and, therefore, allow easier analyses of cause–effect relationship than experiments in whole animals. Obviously, experiments in human cells and in 3D do not solve all problems of cell culture work, including dependence on numerous and only partially controlled culture conditions, reproducibility, and robustness.[70] However, good evidence suggests that cardiac myocytes in a 3D cardiac tissue reach a higher degree of maturation so that 3D tissues are more stable than 2D cultures and allow higher content readouts and automation. The upcoming chapter discusses the current state and direction of further development.

State of the Art

As outlined in previous paragraphs, several methods are available today to create heart muscles and, in combination with hESC or hiPSC, human heart muscles. Of the 3 main tissue engineering approaches, the hydrogel method has been originally designed for in vitro testing.[7] It has gained the widest application in this field because it allows straightforward measurements of force and other parameters of heart muscle function, it is simple, and it can be miniaturized and automated. An interesting alternative is a method to culture cardiac myocytes on strips of elastic foils, which are rhythmically moved as myocytes contract in synchrony. The movement can be monitored by video cameras and reflects contractile force;[71] however, it is not clear to what extent this 2D cell layer approach can be considered a tissue engineering technique.

Models

The principle is that cell–hydrogel mixtures are cast in molds and after solidification are transferred to cell culture dishes. The developing tissues generate force against the anchors they are attached to and, therefore, align in parallel to the force lines. Force can be measured either under nonsterile conditions in an organ bath or repeatedly in a sterile manner by automated video-optical recordings[24] or even continuously. The advantages of fibrin as hydrogel when compared with former ones are several-fold. Because of the fast polymerization, cells cannot concentrate on the bottom, giving a more even cell density. The fast transfer from the casting mold (2 hours when compared with 5 days with collagen) provides faster nutrient and oxygen supply. The developing tissue slightly bends the flexible silicone posts toward each other. This keeps the tissue

under continuous tension and causes a perfect longitudinal alignment. The EHT performs contractile work (auxotonic contractions) against the elastic posts, closely mimicking physiological conditions. Recent work indicates that this has profound biological consequences (see Applications and Disease Modeling section of this article).[72] Finally, the method is simple and allows automated and repeated measurements of contractile function, a requisite for preclinical drug development and other applications. Further simplification and miniaturization of the method to a multiwell format have recently been described (Figure 3A).[73]

Function and Physiological Responses

Engineered 3D constructs are little heart muscles and as such allow measurements of essentially all parameters of heart muscle function. This includes not only twitch force, twitch kinetics, beating rate, and rhythm but also diastolic tension, passive tension, and, importantly, intracellular Ca^{2+} transients.[22] Measurement of Ca^{2+} transients in EHTs is easier than in intact heart muscle preparations, such as papillary muscles, because cardiac myocytes in EHTs readily take up Ca^{2+} indicators, such as Fura-2 AM. Moreover, EHTs are stable for weeks, allowing determination of the development of contractile function over time, as well the effect of experimental manipulations (Figure 3B and 3C). This relates to yet another advantage when compared with intact muscle work. EHTs are easily accessible to genetic manipulation, initially mainly performed with adenovirus[7,22] but today increasingly with adeno-associated virus isotype 6, which provides excellent transduction efficiency and, in contrast to adenovirus, little or no toxicity.

As reviewed recently[74] engineered 3D constructs develop relevant contractile force and behave, to a certain extent, as normal heart muscles. However, reported twitch forces of rat EHTs and similar constructs rarely exceed 2 to 4 mN/mm^2 and those of hPSC-derived 3D engineered tissues were reported to be in the same range when a genetic selection marker was used to enrich for cardiomyocytes after differentiation.[31] Yet, forces from hESC-derived or hiPSC-derived constructs are usually in the range of 0.08 mN/mm^2,[33] to 0.12 mN/mm^2 (Figure 3C).[34] In contrast, forces of intact heart muscles can reach 40 to 80 mN/mm^2.[75] The lower forces in engineered cardiac constructs are mainly explained by lower cardiac myocyte density, lower sarcomere volume fraction, and the overall lower level of cardiac myocyte differentiation.[74]

Cardiac 3D constructs from chickens, rats, mice, and humans show a positive inotropic and chronotropic response to β-adrenergic stimulation, which is sensitive to the muscarinic agonist carbachol.[74] Furthermore, they increase force with

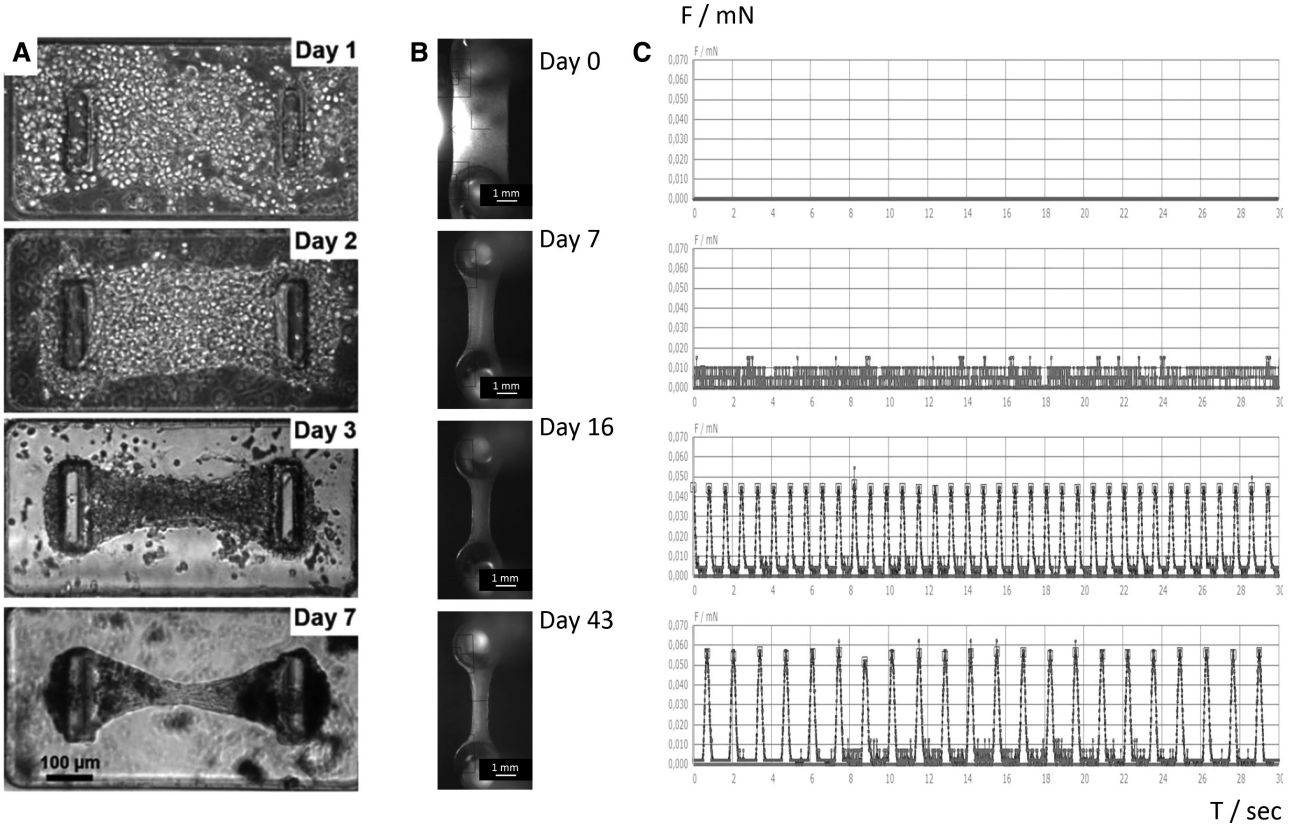

Figure 3. Miniaturized and automated 3-dimensional in vitro test systems. A, Temporal evolution of rat engineered microtissue constructed in fibrin/collagen gels and tethered to cantilevers, produced by photopatterning SU-8 photoresist on silicon wafers. The final tissue length is ≈400 μm and the final diameter is 50 to 70 μm. Reprinted from Boudou et al with permission of the publisher.[73] **B**, Development of human induced pluripotent stem cell (hiPSC)–derived engineered heart tissue (EHT) developing around elastic silicone posts over time, with video-optical view from above. Blue squares indicate positions that are used to analyze EHT contractions. **C**, Corresponding forces over time were determined automatically by video-optical measurements. Of note, spontaneous beating activity is very regular in hiPSC-EHTs. At day 5 to day 7, EHTs start to beat synchronously; at day 10 to day 14, development of force reaches a plateau and remains stable for weeks. **B** and **C**, Unpublished data.

increasing extracellular Ca^{2+} concentrations and show a force–response to increases in length (Frank–Starling behavior) and a force–frequency relationship that depends on the species. In chicken EHTs, it was positive[7]; in rat,[76] murine,[22] and mouse EHTs,[77] it was negative. Collectively, this demonstrates that 3D engineered tissues function qualitatively like native muscle. However, their functional responses to isoprenaline are moderate. Part of this difference is because of an unusually high sensitivity to external Ca^{2+} concentrations. Whereas a nonfailing rat heart muscle increases its force >2-fold when Ca^{2+} is increased from a physiological concentration of ≈2 to 5 mmol/L, EHTs are already maximally active at 1.6 mmol/L.[20] Thus, to see effects of isoprenaline, Ca^{2+} needs to be lowered to the EC_{50} for Ca^{2+}. These data point to immaturity of excitation–contraction coupling in EHTs.

Applications

Until now, most studies concentrated on assay development without actually applying the technology to answer biological questions. Some noteworthy exceptions exist, and the time is right for a more widespread application of the assays available.

1. Regarding target validation and functional genomics. Several years ago, we showed that shRNA-mediated knockdown of protein kinase C-α (by adenovirus gene transfer) improved contractile force under basal and isoprenaline-stimulated conditions,[78] supporting evidence in the literature for a negative inotropic role of protein kinase C-α.[79] More recently, we used the model to overexpress potential disease-causing genetic variants of four and a half LIM domain protein 1, lamin A/C, or cardiac ankyrin repeat protein 1 in EHTs.[80–82] Functional differences induced by the different variants added to a better understanding of their role in disease. The group of Ralphe used ECTs from cardiac myosin-binding protein (cMyBP)-C knockout mice to characterize primary consequences of the HCM-related defect and reintroduced wild-type cMyBP-C by adenoviral overexpression to rescue the phenotype.[22]
2. Test beds for cell therapy are 3D cardiac constructs to study cell–cell interactions with regard to cell therapy. The mechanisms of the beneficial effect of cell infusions/injections into the heart remain incompletely understood. Researchers have used engineered 3D constructs to study this question in vitro under conditions that are easier to control than those of in vivo injections. One study added mesenchymal stem cell to heart tissue constructs that were, compared with control, depleted of 50% cardiac myocytes, mimicking a disease state.[83] This nonmyocyte substitution of myocytes restored force to almost control values, interestingly only for the first 15 days, similar to the transient benefit observed in some cell therapy studies. Others injected ESCs or ESC-derived progenitor cells into 3D engineered tissues and observed both beneficial, paracrine, and unwanted effects, such as conduction block and teratoma formation with undifferentiated ESC.[84]
3. Disease modeling is an exciting new avenue for biomedical research that has been opened by the availability of patient-specific hiPSC lines to model genetic and other cardiac diseases on an individual basis. Both electric and structural inherited cardiac diseases have been studied in hiPSC-derived cardiac myocytes and exhibit key characteristics of the diseases.[85] Yet, the full potential of this technology has not been uncovered and, particularly with structural heart diseases, 3D cardiac constructs will likely help to uncover subtle quantitative differences characteristic for these diseases. HCM, the most frequent inherited cardiac disease with a prevalence of 1:500, is a good example. It is an autosomal-dominant disease on the basis of mutations in sarcomeric genes, with β-myosin heavy chain and cMyBP-C prevailing. First symptoms generally occur relatively late in life (typically during puberty; ie, the hearts of these patients are essentially normal for ≥10 years). HCM has a variable phenotypic expression, ranging from normal life expectancy and little symptoms to sudden cardiac death, atrial fibrillation, severe obstruction, and early contractile failure.[86] Mouse models of HCM show, in the heterozygous state that is typical for human HCM, no or almost no phenotype at all. Thus, it is surprising that isolated cells from patients with heterozygous gene mutations showed full-blown hypertrophy and other parameters of the HCM disease phenotype under standard culture conditions. In contrast, tissue constructs from homozygous cMyBP-C KO mice show only discrete but highly reproducible alterations in contractile function.[22,87] Similarly, EHTs from heterozygous cMyBP-C knock-in mice, a model that reflects human HCM most closely, were completely normal under baseline conditions but showed altered drug responses compatible with increased Ca^{2+} sensitivity of the myofilaments.[88] The first results of hiPSC-derived 3D engineered tissues are eagerly awaited.

Disease is an altered, nonhealthy state of an organism. For example, heart failure is a clinical syndrome defined by signs and symptoms of malperfusion. Therefore, it is disputed how far this state can be modeled in the dish. Yet, key features of the disease can clearly be induced in vitro. We have recently shown that auxotonically contracting rat EHTs that are exposed to markedly increased afterload (by stiffening the silicone posts they are attached to) develop not only cardiac myocyte hypertrophy, a fetal gene expression program, increased collagen deposition, and increased glucose consumption per beat, but also decreased twitch force and prolonged relaxation, all parameters known to occur in human heart failure.[72] This offers interesting perspectives in terms of therapy testing, particularly if similar reactions can be induced in hESC/hiPSC-EHTs (which has yet to be shown). An important conclusion of these data is that static culture conditions (likely both 2D and 3D) induce a baseline disease phenotype, which should be taken into account when interpreting in vitro disease modeling studies.

Drug screening with 3D heart muscles is the most obvious application but it is a difficult application. True drug screening requires not only high content readouts but also miniaturized, automated, and humanized systems (Figure 3A–3C).[24,34,73] Three major applications are envisioned: screening for proarrhythmic, cardiotoxic, and cardiotonic drugs, the first 2 as part of preclinical toxicology, the latter for cardiovascular drug development programs. The major argument to use 3D tissue assays in these applications is the large amount of information that can be retrieved in a human muscle context, not the throughput

that is small-to-medium when compared with professional drug screening assays in which thousands of compounds are tested per day. Until now, only proarrhythmic drugs have been systematically tested in EHTs, both in rats[24] and in human.[34] Some important conclusions can be drawn from these data. First, prolongations of repolarization, the major underlying cause of drug-induced QT-prolongation and torsade-de-pointes arrhythmias in humans, precipitate in EHTs as reductions in relaxation velocity and prolongations of the time of relaxation. Second, rat EHTs are insensitive to specific blockers of rapid delayed rectifier potassium current (human ether-a-go-go-related gene channel, eg, dofetilide or E-4031), which reflects the insignificant role of this current for rodent heart repolarization (our unpublished data). Therefore, they are not suitable for detecting hERG-dependent proarrhythmic effects but are a good test system to detect hERG-independent effects that seem to be more prevalent than anticipated. Third, human EHTs are highly sensitive to hERG blockers. Importantly, and in contrast to hESC cultured in 2D or as embryoid bodies in which much higher concentrations are required to see effects,[89] threshold concentrations for relaxation slowing closely reflect IC_{50} values for hERG channel assays. This suggests that EHTs might be a better model to detect hERG effects. A second application of EHTs is testing for cardiotoxicity, an increasing problem of the so-called targeted anticancer therapy.

Current Developments and Open Questions

Despite the enormous progress made in tissue engineering, assay development and PSC biology for the past decade, it is humbling how comparably little has been gained. Few biological questions have been answered with these models that could have not been studied or could have not been so well-studied with others. Importantly, the pharmaceutical industry remains reserved regarding adapting these in vitro technologies for toxicology and therapy development. In the view of the authors, the time is right for a change, but change requires the following steps.

1. Fully standardized protocols for the derivatization of hiPSC and of cardiac myocytes. Variability between cell lines and batches remains a serious problem when it comes to quantitative measurements, definition of normal values, and SDs. Numerous protocols reporting cell line–independent cardiac differentiation efficacies of ≥80%,[90] as well as methods to purify cardiac myocytes using surface markers such as SIRPA1[91] or lactate containing glucose-free media compositions, certainly represent important progress, but the multitude of protocols suggest already less than perfect robustness.
2. Complete serum-free culture conditions for EHTs or similar constructs. Whereas 3D engineered tissues can be principally cultivated serum-free, as shown for hPSC-constructs,[33] they never reached the same level of maturity and robustness as those cultured in serum-containing media. Identification of the essential serum factors that cause maturation (in addition to triiodothyronine) is an important and urgent task.
3. The maturation state of cardiac myocytes in human EHTs is clearly better than that in 2D or embryoid body culture, but not as good as that in rat EHTs and much less advanced than that of adult human ventricular myocytes. For example, real-time quantitative polymerase chain reaction analyses showed that in transcript levels of myosin regulatory light chain 2, ventricular isoform were >1000× lower in 2D cultures than in the adult human heart when compared with <100-fold lower in EHT (our unpublished data). This is a critical situation, particularly with regard to human disease modeling with hiPSC, which assumes that the effect of a single point mutation in 1 gene (in most prevalent cardiac diseases in the heterozygous state) can be faithfully detected against the thousands of severe abnormalities in the quantitative expression of critical cardiac genes.
4. As outlined in this article, many of the advantages of 3D engineered tissues compared with 2D cultured cells and advantages of assays compared to each other remain largely theoretical. It will be difficult for a laboratory to systematically set-up various test systems and directly compare them. But it is rather easy to answer this important question in an international collaborative effort. A start could be the comparative blinded testing of a limited set of model compounds (eg, 50 proarrhythmic, cardiotoxic, or harmless drugs).

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Disclosures

None.

References

1. Eschenhagen T, Wakatsuki T, Elson EL. A new method to measure isometric force of contraction in embryonic cardiac myocytes. Report No. 95-17 on the Second International Conference on Cellular Engineering, LaJolla August 19–22 (Abstract). 1995.
2. Sawa Y, Miyagawa S, Sakaguchi T, Fujita T, Matsuyama A, Saito A, Shimizu T, Okano T. Tissue engineered myoblast sheets improved cardiac function sufficiently to discontinue LVAS in a patient with DCM: report of a case. *Surg Today*. 2012;42:181–184.
3. Eschenhagen T, Zimmermann WH. Engineering myocardial tissue. *Circ Res*. 2005;97:1220–1231.
4. Moscona AA. Tissues from dissociated cells. *Sci Am.* 1959;200:132–134.
5. Kelm JM, Ehler E, Nielsen LK, Schlatter S, Perriard JC, Fussenegger M. Design of artificial myocardial microtissues. *Tissue Eng*. 2004;10:201–214.
6. Vandenburgh HH, Karlisch P, Farr L. Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel. *In Vitro Cell Dev Biol*. 1988;24:166–174.
7. Eschenhagen T, Fink C, Remmers U, Scholz H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. *FASEB J*. 1997;11:683–694.
8. Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: a new approach to repair the infarcted myocardium? *Circulation*. 2000;102:III56–III61.
9. Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proc Natl Acad Sci U S A*. 2004;101:18129–18134.
10. Li RK, Jia ZQ, Weisel RD, Mickle DA, Choi A, Yau TM. Survival and function of bioengineered cardiac grafts. *Circulation*. 1999;100:II63–II69.
11. Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjak-Novakovic G. Cardiac tissue engineering: cell seeding,

cultivation parameters, and tissue construct characterization. *Biotechnol Bioeng*. 1999;64:580–589.
12. Lesman A, Habib M, Caspi O, Gepstein A, Arbel G, Levenberg S, Gepstein L. Transplantation of a tissue-engineered human vascularized cardiac muscle. *Tissue Eng Part A*. 2010;16:115–125.
13. Marsano A, Maidhof R, Wan LQ, Wang Y, Gao J, Tandon N, Vunjak-Novakovic G. Scaffold stiffness affects the contractile function of three-dimensional engineered cardiac constructs. *Biotechnol Prog*. 2010;26:1382–1390.
14. Dvir T, Timko BP, Brigham MD, Naik SR, Karajanagi SS, Levy O, Jin H, Parker KK, Langer R, Kohane DS. Nanowired three-dimensional cardiac patches. *Nat Nanotechnol*. 2011;6:720–725.
15. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med*. 2008;14:213–221.
16. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ Res*. 2002;90:e40.
17. L'Heureux N, Pâquet S, Labbé R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. *FASEB J*. 1998;12:47–56.
18. Kubo H, Shioyama T, Oura M, Suzuki A, Ogawa T, Makino H, Takeda S, Kino-Oka M, Shimizu T, Okano T, Yamamori S. Development of automated 3-dimensional tissue fabrication system tissue factory—automated cell isolation from tissue for regenerative medicine. *Conf Proc IEEE Eng Med Biol Soc*. 2013;2013:358–361.
19. Baar K, Birla R, Boluyt MO, Borschel GH, Arruda EM, Dennis RG. Self-organization of rat cardiac cells into contractile 3-D cardiac tissue. *FASEB J*. 2005;19:275–277.
20. Zimmermann WH, Schneiderbanger K, Schubert P, Didié M, Münzel F, Heubach JF, Kostin S, Neuhuber WL, Eschenhagen T. Tissue engineering of a differentiated cardiac muscle construct. *Circ Res*. 2002;90:223–230.
21. Black LD 3rd, Meyers JD, Weinbaum JS, Shvelidze YA, Tranquillo RT. Cell-induced alignment augments twitch force in fibrin gel-based engineered myocardium via gap junction modification. *Tissue Eng Part A*. 2009;15:3099–3108.
22. de Lange WJ, Hegge LF, Grimes AC, Tong CW, Brost TM, Moss RL, Ralphe JC. Neonatal mouse-derived engineered cardiac tissue: a novel model system for studying genetic heart disease. *Circ Res*. 2011;109:8–19.
23. Fink C, Ergün S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J*. 2000;14:669–679.
24. Hansen A, Eder A, Bonstrup M, Flato M, Mewe M, Schaaf S, Aksehirlioglu B, Schworer A, Uebeler J, Eschenhagen T. Development of a drug screening platform based on engineered heart tissue. *Circ Res*. 2010;107:35–44.
25. Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med*. 2006;12:452–458.
26. Nunes SS, Miklas JW, Liu J, et al. Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nat Methods*. 2013;10:781–787.
27. Jugdutt BI. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? *Circulation*. 2003;108:1395–1403.
28. Banerjee I, Fuseler JW, Price RL, Borg TK, Baudino TA. Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am J Physiol Heart Circ Physiol*. 2007;293:H1883–H1891.
29. Naito H, Melnychenko I, Didié M, Schneiderbanger K, Schubert P, Rosenkranz S, Eschenhagen T, Zimmermann WH. Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. *Circulation*. 2006;114:I72–I78.
30. Radisic M, Marsano A, Maidhof R, Wang Y, Vunjak-Novakovic G. Cardiac tissue engineering using perfusion bioreactor systems. *Nat Protoc*. 2008;3:719–738.
31. Kensah G, Roa Lara A, Dahlmann J, et al. Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. *Eur Heart J*. 2013;34:1134–1146.
32. Stevens KR, Kreutziger KL, Dupras SK, Korte FS, Regnier M, Muskheli V, Nourse MB, Bendixen K, Reinecke H, Murry CE. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. *Proc Natl Acad Sci U S A*. 2009;106:16568–16573.
33. Tulloch NL, Muskheli V, Razumova MV, Korte FS, Regnier M, Hauch KD, Pabon L, Reinecke H, Murry CE. Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circ Res*. 2011;109:47–59.
34. Schaaf S, Shibamiya A, Mewe M, Eder A, Stöhr A, Hirt MN, Rau T, Zimmermann WH, Conradi L, Eschenhagen T, Hansen A. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. *PLoS One*. 2011;6:e26397.
35. Zeng QC, Guo Y, Liu L, Zhang XZ, Li RX, Zhang CQ, Hao QX, Shi CH, Wu JM, Guan J. Cardiac fibroblast-derived extracellular matrix produced in vitro stimulates growth and metabolism of cultured ventricular cells. *Int Heart J*. 2013;54:40–44.
36. Sekine H, Shimizu T, Hobo K, Sekiya S, Yang J, Yamato M, Kurosawa H, Kobayashi E, Okano T. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation*. 2008;118:S145–S152.
37. Tiburcy M, Didie M, Boy O, et al. Terminal differentiation, advanced organotypic maturation, and modeling of hypertrophic growth in engineered heart tissue. *Circ Res*. 2011;109:1105–1114.
38. Pritchett-Corning KR. Euthanasia of neonatal rats with carbon dioxide. *J Am Assoc Lab Anim Sci*. 2009;48:23–27.
39. Vollert I, Seiffert M, Bachmair J, Sander M, Eder A, Conradi L, Vogelsang A, Schulze T, Uebeler J, Holnthoner W, Redl H, Reichenspurner H, Hansen A, Eschenhagen T. In-vitro perfusion of engineered heart tissue through endothelialized channels. *Tissue Eng Part A*. December 11, 2013. doi:10.1089/ten.tea.2013.0214: http://online.liebertpub.com/doi/full/10.1089/ten.tea.2013.0214. Accessed January 3, 2014.
40. Dow J, Simkhovich BZ, Kedes L, Kloner RA. Washout of transplanted cells from the heart: a potential new hurdle for cell transplantation therapy. *Cardiovasc Res*. 2005;67:301–307.
41. Yildirim Y, Naito H, Didié M, Karikkineth BC, Biermann D, Eschenhagen T, Zimmermann WH. Development of a biological ventricular assist device: preliminary data from a small animal model. *Circulation*. 2007;116:I16–I23.
42. Yost MJ, Baicu CF, Stonerock CE, Goodwin RL, Price RL, Davis JM, Evans H, Watson PD, Gore CM, Sweet J, Creech L, Zile MR, Terracio L. A novel tubular scaffold for cardiovascular tissue engineering. *Tissue Eng*. 2004;10:273–284.
43. Sekine H, Shimizu T, Yang J, Kobayashi E, Okano T. Pulsatile myocardial tubes fabricated with cell sheet engineering. *Circulation*. 2006;114:I87–I93.
44. Seif-Naraghi SB, Singelyn JM, Salvatore MA, et al. Safety and efficacy of an injectable extracellular matrix hydrogel for treating myocardial infarction. *Sci Transl Med*. 2013;5:173ra125.
45. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science*. 2009;324:98–102.
46. Landa N, Miller L, Feinberg MS, Holbova R, Shachar M, Freeman I, Cohen S, Leor J. Effect of injectable alginate implant on cardiac remodeling and function after recent and old infarcts in rat. *Circulation*. 2008;117:1388–1396.
47. Godier-Furnémont AF, Martens TP, Koeckert MS, Wan L, Parks J, Arai K, Zhang G, Hudson B, Homma S, Vunjak-Novakovic G. Composite scaffold provides a cell delivery platform for cardiovascular repair. *Proc Natl Acad Sci U S A*. 2011;108:7974–7979.
48. Davis ME, Hsieh PC, Grodzinsky AJ, Lee RT. Custom design of the cardiac microenvironment with biomaterials. *Circ Res*. 2005;97:8–15.
49. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest*. 2005;115:572–583.
50. Anderl JN, Robey TE, Stayton PS, Murry CE. Retention and biodistribution of microspheres injected into ischemic myocardium. *J Biomed Mater Res A*. 2009;88:704–710.
51. Bian W, Liau B, Badie N, Bursac N. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. *Nat Protoc*. 2009;4:1522–1534.
52. Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T. Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation*. 2002;106:I151–I157.
53. Furuta A, Miyoshi S, Itabashi Y, Shimizu T, Kira S, Hayakawa K, Nishiyama N, Tanimoto K, Hagiwara Y, Satoh T, Fukuda K, Okano T, Ogawa S. Pulsatile cardiac tissue grafts using a novel three-dimensional cell sheet manipulation technique functionally integrates with the host heart, in vivo. *Circ Res*. 2006;98:705–712.
54. Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T. Polysurgery of cell sheet grafts overcomes

diffusion limits to produce thick, vascularized myocardial tissues. *FASEB J*. 2006;20:708–710.
55. Stevens KR, Pabon L, Muskheli V, Murry CE. Scaffold-free human cardiac tissue patch created from embryonic stem cells. *Tissue Eng Part A*. 2009;15:1211–1222.
56. Tee R, Morrison WA, Dusting GJ, Liu GS, Choi YS, Hsiao ST, Dilley RJ. Transplantation of engineered cardiac muscle flaps in syngeneic rats. *Tissue Eng Part A*. 2012;18:1992–1999.
57. Sekine H, Shimizu T, Sakaguchi K, Dobashi I, Wada M, Yamato M, Kobayashi E, Umezu M, Okano T. In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. *Nat Commun.* 2013;4:1399.
58. Chiu LL, Montgomery M, Liang Y, Liu H, Radisic M. Perfusable branching microvessel bed for vascularization of engineered tissues. *Proc Natl Acad Sci U S A*. 2012;109:E3414–E3423.
59. Sakaguchi K, Shimizu T, Horaguchi S, Sekine H, Yamato M, Umezu M, Okano T. In vitro engineering of vascularized tissue surrogates. *Sci Rep.* 2013;3:1316.
60. Reinecke H, Poppa V, Murry CE. Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. *J Mol Cell Cardiol*. 2002;34:241–249.
61. Kohl P, Camelliti P. Cardiac myocyte-nonmyocyte electrotonic coupling: implications for ventricular arrhythmogenesis. *Heart Rhythm*. 2007;4:233–235.
62. Caspi O, Huber I, Kehat I, Habib M, Arbel G, Gepstein A, Yankelson L, Aronson D, Beyar R, Gepstein L. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol*. 2007;50:1884–1893.
63. Chen QZ, Ishii H, Thouas GA, Lyon AR, Wright JS, Blaker JJ, Chrzanowski W, Boccaccini AR, Ali NN, Knowles JC, Harding SE. An elastomeric patch derived from poly(glycerol sebacate) for delivery of embryonic stem cells to the heart. *Biomaterials*. 2010;31:3885–3893.
64. Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T, Sawa Y. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation*. 2012;126:S29–S37.
65. Swijnenburg RJ, Schrepfer S, Govaert JA, Cao F, Ransohoff K, Sheikh AY, Haddad M, Connolly AJ, Davis MM, Robbins RC, Wu JC. Immunosuppressive therapy mitigates immunological rejection of human embryonic stem cell xenografts. *Proc Natl Acad Sci U S A*. 2008;105:12991–12996.
66. Zhao T, Zhang ZN, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. *Nature*. 2011; 474:212–215.
67. Guha P, Morgan JW, Mostoslavsky G, Rodrigues NP, Boyd AS. Lack of immune response to differentiated cells derived from syngeneic induced pluripotent stem cells. *Cell Stem Cell*. 2013;12:407–412.
68. Didie M, Christalla P, Rubart M, et al. Parthenogenetic stem cells for tissue-engineered heart repair. *J Clin Invest*. 2013;123:1285–1298.
69. Robertson EJ, Evans MJ, Kaufman MH. X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. *J Embryol Exp Morphol*. 1983;74:297–309.
70. Rajamohan D, Matsa E, Kalra S, Crutchley J, Patel A, George V, Denning C. Current status of drug screening and disease modelling in human pluripotent stem cells. *Bioessays*. 2013;35:281–298.
71. Feinberg AW, Feigel A, Shevkoplyas SS, Sheehy S, Whitesides GM, Parker KK. Muscular thin films for building actuators and powering devices. *Science*. 2007;317:1366–1370.
72. Hirt MN, Sörensen NA, Bartholdt LM, Boeddinghaus J, Schaaf S, Eder A, Vollert I, Stöhr A, Schulze T, Witten A, Stoll M, Hansen A, Eschenhagen T. Increased afterload induces pathological cardiac hypertrophy: a new in vitro model. *Basic Res Cardiol*. 2012;107:307.
73. Boudou T, Legant WR, Mu A, Borochin MA, Thavandiran N, Radisic M, Zandstra PW, Epstein JA, Margulies KB, Chen CS. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. *Tissue Eng Part A*. 2012;18:910–919.
74. Eschenhagen T, Eder A, Vollert I, Hansen A. Physiological aspects of cardiac tissue engineering. *Am J Physiol Heart Circ Physiol*. 2012;303:H133–H143.
75. van der Velden J, Klein LJ, van der Bijl M, Huybregts MA, Stooker W, Witkop J, Eijsman L, Visser CA, Visser FC, Stienen GJ. Force production in mechanically isolated cardiac myocytes from human ventricular muscle tissue. *Cardiovasc Res*. 1998;38:414–423.
76. Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnol Bioeng*. 2000;68:106–114.
77. Liau B, Christoforou N, Leong KW, Bursac N. Pluripotent stem cell-derived cardiac tissue patch with advanced structure and function. *Biomaterials*. 2011;32:9180–9187.
78. El-Armouche A, Singh J, Naito H, Wittköpper K, Didié M, Laatsch A, Zimmermann WH, Eschenhagen T. Adenovirus-delivered short hairpin RNA targeting PKCalpha improves contractile function in reconstituted heart tissue. *J Mol Cell Cardiol*. 2007;43:371–376.
79. Liu Q, Molkentin JD. Protein kinase calpha as a heart failure therapeutic target. *J Mol Cell Cardiol*. 2011;51:474–478
80. Friedrich FW, Wilding BR, Reischmann S, et al. Evidence for fhl1 as a novel disease gene for isolated hypertrophic cardiomyopathy. *Hum Mol Genet*. 2012;21:3237–3254.
81. Cattin ME, Bertrand AT, Schlossarek S, et al. Heterozygous lmnadelk32 mice develop dilated cardiomyopathy through a combined pathomechanism of haploinsufficiency and peptide toxicity. *Hum Mol Genet*. 2013;22:3152–3164.
82. Crocini C, Arimura T, Reischmann S, Eder A, Braren I, Hansen A, Eschenhagen T, Kimura A, Carrier L. Impact of ANKRD1 mutations associated with hypertrophic cardiomyopathy on contraction parameters of engineered heart tissue. *Basic Res Cardiol*. 2013;108:349.
83. Serrao GW, Turnbull IC, Ancukiewicz D, Kim do E, Kao E, Cashman TJ, Hadri L, Hajjar RJ, Costa KD. Myocyte-depleted engineered cardiac tissues support therapeutic potential of mesenchymal stem cells. *Tissue Eng Part A*. 2012;18:1322–1333.
84. Dengler J, Song H, Thavandiran N, Massé S, Wood GA, Nanthakumar K, Zandstra PW, Radisic M. Engineered heart tissue enables study of residual undifferentiated embryonic stem cell activity in a cardiac environment. *Biotechnol Bioeng*. 2011;108:704–719.
85. Mercola M, Colas A, Willems E. Induced pluripotent stem cells in cardiovascular drug discovery. *Circ Res*. 2013;112:534–548.
86. Maron BJ, Maron MS. Hypertrophic cardiomyopathy. *Lancet*. 2013;381:242–255.
87. de Lange WJ, Grimes AC, Hegge LF, Ralphe JC. Ablation of cardiac myosin-binding protein-C accelerates contractile kinetics in engineered cardiac tissue. *J Gen Physiol*. 2013;141:73–84.
88. Stöhr A, Friedrich FW, Flenner F, Geertz B, Eder A, Schaaf S, Hirt MN, Uebeler J, Schlossarek S, Carrier L, Hansen A, Eschenhagen T. Contractile abnormalities and altered drug response in engineered heart tissue from Mybpc3-targeted knock-in mice. *J Mol Cell Cardiol*. 2013;63:189–198.
89. Braam SR, Tertoolen L, van de Stolpe A, Meyer T, Passier R, Mummery CL. Prediction of drug-induced cardiotoxicity using human embryonic stem cell-derived cardiomyocytes. *Stem Cell Res*. 2010;4:107–116.
90. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell*. 2011;8:228–240.
91. Dubois NC, Craft AM, Sharma P, Elliott DA, Stanley EG, Elefanty AG, Gramolini A, Keller G. SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nat Biotechnol.* 2011;29:1011–1018.
92. Streckfuss-Bomeke K, Wolf F, Azizian A, et al. Comparative study of human-induced pluripotent stem cells derived from bone marrow cells, hair keratinocytes, and skin fibroblasts. *Eur Heart J*. 2013;34:2618–2629.
93. Mühlhäuser U, Zolk O, Rau T, Münzel F, Wieland T, Eschenhagen T. Atorvastatin desensitizes beta-adrenergic signaling in cardiac myocytes via reduced isoprenylation of G-protein gamma-subunits. *FASEB J*. 2006;20:785–787.
94. Matsuura K, Masuda S, Haraguchi Y, Yasuda N, Shimizu T, Hagiwara N, Zandstra PW, Okano T. Creation of mouse embryonic stem cell-derived cardiac cell sheets. *Biomaterials*. 2011;32:7355–7362.
95. Kelm JM, Djonov V, Hoerstrup SP, Guenter CI, Ittner LM, Greve F, Hierlemann A, Sanchez-Bustamante CD, Perriard JC, Ehler E, Fussenegger M. Tissue-transplant fusion and vascularization of myocardial microtissues and macrotissues implanted into chicken embryos and rats. *Tissue Eng*. 2006;12:2541–2553.