Tissue Engineering of a Differentiated Cardiac Muscle Construct


Abstract—Cardiac tissue engineering is an emerging field. The suitability of engineered heart tissue (EHT) for both in vitro and in vivo applications will depend on the degree of syncytoid tissue formation and cardiac myocyte differentiation in vitro, contractile function, and electrophysiological properties. Here, we demonstrate that cardiac myocytes from neonatal rats, when mixed with collagen I and matrix factors, cast in circular molds, and subjected to phasic mechanical stretch, reconstitute ring-shaped EHTs that display important hallmarks of differentiated myocardium. Comparative histological analysis of EHTs with native heart tissue from newborn, 6-day-old, and adult rats revealed that cardiac cells in EHTs reconstitute intensively interconnected, longitudinally oriented, cardiac muscle bundles with morphological features resembling adult rather than immature native tissue. Confocal and electron microscopy demonstrated characteristic features of native differentiated myocardium; some of these features are absent in myocytes from newborn rats: (1) highly organized sarcomeres in registry; (2) adherens junctions, gap junctions, and desmosomes; (3) a well-developed T-tubular system and dyad formation with the sarcoplasmic reticulum; and (4) a basement membrane surrounding cardiac myocytes. Accordingly, EHTs displayed contractile characteristics of native myocardium with a high ratio of twitch (0.4 to 0.8 mN) to resting tension (0.1 to 0.3 mN) and a strong β-adrenergic inotropic response. Action potential recordings demonstrated stable resting membrane potentials of −66 to −78 mV, fast upstroke kinetics, and a prominent plateau phase. The data indicate that EHTs represent highly differentiated cardiac tissue constructs, making EHTs a promising material for in vitro studies of cardiac function and tissue replacement therapy. (Circ Res. 2002;90:223-230.)

Key Words cell culture ■ growth and development ■ morphology ■ isometric contraction ■ ultrastructure

Engineering of 3D cardiac tissue constructs in vitro offers new perspectives for basic cardiovascular research and for tissue replacement therapy.1–6 Whereas some groups have reported the spontaneous formation of 3D heart cell aggregates with a diameter of 100 to 300 μm when embryonic chick7 or neonatal rat cardiac myocytes8 were subjected to gyrated shaking or microgravity, most groups use scaffold proteins (eg, collagen or gelatin) or synthetic polymers (eg, alginate or polyglycolic acid) for tissue reconstitution from isolated cells. The latter allows for the design of tailored geometric forms. Decker et al8 applied alginate matrices to reconstitute cardiac myocytes from adult cats. Despite the conservation of the classic rod shape of adult cardiac myocytes in this culture form, the reestablishment of a contracting syncytoid tissue was not observed. In contrast, immature cells from embryonic chicken and from fetal or neonatal rats appear to have the capacity to reconstitute tissue-like structures of different shapes and sizes when they are cultured within a scaffold substratum.1–5 Most of these constructs exhibited spontaneous contractile activity, and one technique originally developed by our group (engineered heart tissue [EHT]) allowed direct force measurement under isometric conditions by growing the reconstituted tissue between two hook-and-loop fastener (Velcro)-coated tubes.1,2 Yet, the present techniques have a number of shortcomings that limit their usefulness for both in vitro and in vivo application. (1) Regarding technical aspects, the original EHT technique used a rather complicated casting procedure, in which Velcro-coated glass or silicone tubes had to be produced by hand, could only be reused 5 to 10 times, had to be assembled in casting molds with metal spacers, and (because of inevitable variations in size) gave rise to EHTs of varying size and quality. (2) Regarding tissue macroscopic and functional quality, the original EHT lattices exhibited an inhomo- geneous cell distribution with good tissue formation at the free edges and a loose network of disoriented cells in the...
center. This has contributed to differences in passive forces within the tissue construct. The influence of active or passive force on cardiac myocyte growth, morphology, orientation, mitogen-activated protein kinase activation, and gene expression has been demonstrated by various groups. Accordingly, phasic stretch of planar EHTs induced hypertrophic growth and marked functional improvement. Yet, the principle inhomogeneity remained and most likely accounted for resting tension (RT) to be much higher than twitch tension (TT) in the EHT lattices. This not only is in marked contrast to native heart tissue (in which TT is generally higher than RT) but also limits its usefulness as a tissue graft for replacement therapy, because only a minor fraction of the implanted material would be accessible for direct tissue-to-tissue contact after implantation. Similar problems are likely to be inherent in the other techniques described, in which only a minor fraction of the 3D structure consists of cardiac tissue. (3) Regarding the degree of cardiac myocyte differentiation, cardiac myocytes cultured in the standard 2D culture with the presence of growth-promoting medium conditions (eg, serum and growth supplements) have the tendency to dedifferentiate and to be overgrown by nonmyocytes. Whereas the latter problem appears to be principally overcome in the 3D environment, it remains to be determined whether cardiac myocytes dedifferentiate in the 3D environment provided by collagen I or whether they differentiate and (if so) to which degree the differentiation progresses.

The present study was aimed to develop an improved technique for cardiac tissue engineering in terms of technical feasibility, tissue homogeneity, and cardiac myocyte differentiation. This goal was reached by casting EHTs not as the original lattices but as rings. Surprisingly, cardiac myocytes, when cultured in this system, not only regained histomorphological characteristics of the tissue from which they were derived (hearts from newborn rats) but also surpassed this degree of cardiac differentiation.

Materials and Methods

Engineered Heart Tissue

Circular EHTs were prepared by mixing freshly isolated cardiac myocytes from neonatal rats with collagen type I prepared from rat tails, a basement membrane protein mixture (Matrigel, Becton Dickinson), and concentrated serum–containing culture medium (DMEM, 20% horse serum, 4% chick embryo extract, 200 µg/mL penicillin, and 200 µg/mL streptomycin); pH was neutralized by titration with NaOH (0.1N; see online Table 1 in the data supplement available at http://www.circresaha.org; see online video sequence). Culture medium was changed every day.

Casting and Culture of Circular EHTs

Circular EHTs were cast easily in large series. On average, we reconstituted 30 EHTs from 30 neonatal rat hearts. During

Results

Casting and Culture of Circular EHTs

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Figure 1. Experimental setup for EHT preparation, culture, and analysis of contractile function. a, Casting mold assembly. Silicone tubing (T) was glued to the surface of glass culture dishes. Either Teflon disks (D) or cylinders (C) can be placed over silicone tubing to function as removable spacers during casting mold preparation and EHT culture, respectively. b, EHT condensation around the central Teflon cylinder in casting molds between culture days 1 to 4. After condensation, no change of gross morphology was observed. c, EHTs after transfer in a stretch apparatus to continue culture under unidirectional and cyclic stretch (10%, 2 Hz). d, EHT in a thermostated organ bath. Isometric force was measured in Tyrode’s solution under pulse-field stimulation (37°C, 2 Hz; 5 ms, and 80 to 100 mA). Bars=10 mm.

Force Measurement and Action Potential Recordings

After 14 days (ie, 7 days in casting molds followed by 7 days of stretch), EHTs were transferred into thermostated organ baths and subjected to isometric force measurement as described previously (Figure 1d; see online video sequence). Action potentials were elicited by field stimulation at 1 Hz and recorded with conventional intracellular microelectrodes at 36°C.

Morphological Studies

EHTs were fixed in 4% formaldehyde/1% methanol or 2.5% glutaraldehyde in PBS for light/laser scanning microscopy and transmission electron microscopy (TEM), respectively. After an overnight wash in PBS, EHTs were further processed for light microscopy of hematoxylin and eosin (H&E)-stained paraffin sections, for confocal laser scanning microscopy (CLSM) of immunolabeled vibratome sections or whole-mount samples, and for TEM of contrasted ultrathin sections. Cardiac myocyte morphology within EHT was compared with H&E-stained paraffin sections from native myocardium of newborn rats (postpartum day [dpp] 0), neonatal rats (dpp 6), and adult rats (300 g).

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.
culture, EHTs condensed around the removable central poly-tetrafluoroethylene (Teflon) cylinder within the casting molds (Figure 1b). First contractions of single cells were noted after 24 hours; synchronous contractions of cell clusters started at day 2. Their size increased until the entire EHT beat synchronously (≈1 Hz, day 4 or 5). Over time, contraction became more regular, more vigorous, and faster (≈2 Hz). Physical stability to allow manual handling and mechanical stretch without inflicting damage to the EHT structure was reached after 6 or 7 days in culture. Vigorous spontaneous contractions of EHTs were noted when the stretch device was turned off and after transfer of EHTs into a culture dish (see online video sequence). At this stage, EHT weighed 29.2±1.4 mg (n=12) and had a diameter of 833±17 μm (n=16).

**Histology**

In planar EHT lattices, cardiac myocytes were mainly concentrated at the lateral free edges. In contrast, serial sections of paraffin-embedded circular EHTs (n=7) did not reveal a spatial preference of cell distribution. Complexes of multicellular aggregates and longitudinally oriented cell bundles mainly consisting of cardiac myocytes (Figure 2a) were found throughout circular EHT. The width of these muscle bundles ranged from 30 to 100 μm. For comparison, paraffin sections of native heart tissue from newborn, neonatal, and adult rats (300 g) were investigated (Figures 2b through 2d). In the adult myocardium, compared with the immature tissues, myocytes were larger in width and length, were more intensely stained with eosin, and exhibited clear cross striation, indicating a higher content of myofilaments. Density of myocyte and nonmyocyte nuclei was ≈3-fold lower in the adult tissue, and myocyte nuclei were elongated (length to width 5:1) in contrast to round or oval nuclei in the immature tissue. Surprisingly, histological features of myocytes forming EHTs resembled those of myocytes within native differenti-entiated myocardium. The intensity of eosin staining was much higher than that in the immature tissues; cross striation was visible, albeit to a lesser degree than in the adult tissue; and nuclei had a length/width ratio of 5 to 6:1. Differences from the adult tissue were the smaller absolute size of cardiac myocytes and myocyte nuclei and a less compact overall structure.

**Immunofluorescent Characterization of EHT**

To analyze the overall composition and spatial distribution of cell species within EHTs, vibratome sections were immuno-labeled to identify cardiac myocytes (α-sarcomeric actin, a), smooth muscle cells (α-smooth muscle actin, d), fibroblasts (prolyl-4-hydroxylase, g), and macrophages (ED2-antigen, j). Actin filaments were labeled with phallloidin-TRITC (red; b, e, h, and k). Superposition of images (c, f, i, and l) yields a yellow signal where the double-fluorescent labeling was achieved. Bar=100 μm.

**Figure 3.** Immunolabeling of distinct cell species within EHT. Cellular composition and localization were investigated by immunolabeling (green) of cardiac myocytes (α-sarcomeric actin, a), smooth muscle cells (α-smooth muscle actin, d), fibroblasts (prolyl-4-hydroxylase, g), and macrophages (ED2-antigen, j). Actin filaments were labeled with phallloidin-TRITC (red; b, e, h, and k). Superposition of images (c, f, i, and l) yields a yellow signal where the double-fluorescent labeling was achieved. Bar=100 μm.
technique revealed cell strands (Figure 3) forming a network of intensively interconnected cell bundles throughout the entire EHT that (at variable positions inside the EHT) condensed to solid muscle bundles, as depicted in Figure 2a. High-power CLSM demonstrated that the majority of cell bundles were composed of cardiac myocytes with a high degree of sarcomeric organization (Figure 4b and 4c). At high magnification, capillary structures positive for CD31 (a platelet and endothelial cell adhesion molecule) were noted (Figure 4d).

Ultrastructural Characterization of EHT

Ultrastructural hallmarks of cardiac myocyte differentiation are M-band formation, development of T tubules with dyads/triads, specialized cell-cell junctions, and the reestablishment of an extracellular basement membrane. Most, but not all, of these features were present in the majority of cells (Figures 5 and 6). Cardiac myocytes within EHTs displayed a predominant orientation of sarcomeres in registry along the longitudinal cell axis (Figure 5a). Cross sections of EHT revealed that most cardiac myocytes were densely packed with myofibrils and mitochondria (Figure 5b). Morphometric evaluation of 20 longitudinally oriented, mononucleated cardiac myocytes from 4 EHTs revealed volume fractions as follows: myofibrils (44.7 ± 1.9%), mitochondria (23.9 ± 1.2%), and nucleus (8.9 ± 0.9%). The rest of the cardiac myocyte volume (22.5 ± 1.8%) was occupied by sarcoplasmic reticulum (SR), cytoplasm, and undefined structures. Sarcomeres were composed of Z, I, A, and H bands in most investigated cells. Immature M bands were noted frequently but not in all sarcomeres. If present (Figure 6a), they were clearly less developed than those in adult myocytes, indicating that cardiac myocytes in EHTs exhibit a high, but not a terminal, degree of differentiation. T tubules were observed at the Z-band level (Figures 6b through 6d) and often formed dyads with the SR (Figures 6c and 6d). Specialized cell-cell junctions responsible for mechanical and electrical coupling of cardiac myocytes (adherens junctions, desmosomes, and gap junctions) were found throughout EHTs (Figures 6d and 6e). Cardiac myocytes often formed a well-developed basement membrane as an additional indication of cardiac myocyte integrity (Figure 6f). Atrial secretory granules characteristic for atrial or undifferentiated ventricular myocytes were absent.

TEM provided additional evidence that EHTs are reconstituted from various cell species apart from cardiac myocytes, resembling an organoid cardiac tissue construct (Figure 7). These cells did not populate EHTs in a random fashion but formed distinct structures. The outer surface of EHTs was lined with multiple cell layers consisting mainly of nonmyocytes (fibroblasts, smooth muscle cells, endothelial cells, and...
macrophages; Figure 7a). Fibroblasts, sometimes clearly demonstrating secretory activity, were found throughout EHTs (Figure 7b and also Figures 3g through 3i). Endothelial cells formed characteristic capillary structures that corresponded to CD31-positive cells observed by CLSM (Figure 7c and Figure 4d). Cell debris was frequently sequestrated by macrophages (Figure 7d).

**Contractile Properties of Circular EHTs**

Contractile force and twitch kinetics of electrically stimulated EHTs were investigated under isometric conditions. At the length of maximal force development, TT amounted to 0.36±0.06 mN at an RT of 0.27±0.03 mN. Contraction and relaxation time were 83±2 ms and 154±9 ms, respectively. An increase in extracellular calcium enhanced TT from 0.34±0.06 to 0.75±0.11 mN, with a maximal inotropic response at 1.6 mmol/L (Figure 8, top left); RT and twitch kinetics remained unchanged. β-Adrenergic stimulation induced a maximal increase of TT from 0.28±0.06 to 0.69±0.09 mN at 1 μmol/L isoprenaline (Figure 8, top right). Additionally, isoprenaline shortened the contraction time from 86±4 to 56±2 ms and the relaxation time from 144±8 to 83±3 ms and reduced RT from 0.15±0.02 to 0.05±0.02 mN (Figure 8, bottom panels). The decrease in RT may be mediated by smooth muscle cells that line the surface of EHTs (Figures 3d through 3f). Long-term treatment with growth factors altered functional properties, indicating the general applicability of EHTs as a model of cardiac hypertrophy (see online Table 2 in the data supplement available at http://www.circresaha.org).

**Action Potentials**

After equilibration in Tyrode’s solution, EHT preparations generated only very infrequent spontaneous action potentials. Electrical stimulation at 1 Hz elicited regular action potentials with fast upstroke velocity (dV/dt_{max} 66±8 V/s), an amplitude of 109±2 mV, and a prominent plateau phase with action potential duration at

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** TEM of sarcomeric structures, cell-cell junctions, and basal membrane of cardiac myocytes in EHT. a, Formation of (immature) M bands was noted in some but not all cardiac myocytes, whereas Z, I, A, and H bands were clearly distinguishable in most cardiac myocytes in EHT. b through d, T tubules (TT) of various diameters are shown. Dyad formation with SR was found frequently, especially at the Z-band level. d and e, Gap junctions (GJ), desmosomes (D), and adherens junctions (AJ) interconnect 3D reconstituted cardiac myocytes. f, Well-developed basement membrane (BM) around reconstituted cardiac myocytes. Bars=1 μm.

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** TEM of various cell types found to populate EHT. a, Macrophages (MP), fibroblasts (FB), and other cells with a low degree of differentiation lined the outer surface of EHTs. Underneath, a cell layer mainly consisting of cardiac myocytes (CM) and smooth muscle cells (SMC) can be observed. b, Fibroblasts (FB) with and without signs of secretory activities were found throughout EHT. c, Endothelial cells (EC) were noted to form capillary structures within EHT. d, Cells of leukocytotic origin, such as macrophages and other mononucleated cells (MC, b), were found mainly at the EHT surface but also within EHT. Bars=1 μm.
20%, 50%, and 90% repolarization being 52±2, 87±4, and 148±3 ms, respectively (see online Figure 1 in the data supplement available at http://www.circresaha.org). In all 6 experiments, resting potential (~73±2 mV) was stable during electrical diastole.

**Discussion**

The present study describes a new method for engineering a cardiac tissue–like construct in vitro (EHT). Compared with former systems, EHTs exhibit a better cardiac tissue/matrix ratio, improved contractile function, and a high degree of cardiac myocyte differentiation. Additionally, action potential recordings revealed electrophysiological properties typical of cardiac tissue. The culture as 3D rings is simple, does not require special equipment, and can therefore be performed in any cell culture laboratory. Importantly, the circular culture form allows for future miniaturization and automation.

**In Vitro Applications**

The main advantage of EHTs in our view is that cardiac myocytes in EHTs resemble cardiac myocytes in the intact heart more closely than do those in standard 2D culture systems. This interpretation is supported by the following findings: (1) The cells form a 3D network of intensely interconnected, strictly longitudinally oriented, and electrically and mechanically coupled bundles that resemble loose cardiac tissue. (2) They are apparently exposed to a homogeneous load. The latter feature has not been proven directly (and it would be difficult to do so), but the fact that the cellular network in EHTs was strictly longitudinally oriented and the geometry of a ring both argue for a homogeneous load. As a consequence, tissue formation was much more homogeneous in circular EHTs than in the previously used planar EHT lattices. 

Other researchers did not systematically evaluate this question. 

(3) In accordance with the organized tissue–like morphology, circular EHTs exhibited a tissue-like ratio of TT to RT of 1.33, 3.29, and 14.02 under basal, maximal calcium, and maximal isoprenaline concentrations, respectively. The basal values are in line with similar ratios in intact trabeculae or papillary muscles from humans and rats, indicating that in circular EHTs, the matrix contributes significantly less to mechanic properties than in the planar lattices, for which we have described a ratio of TT to RT of 0.2 to 0.3. 

(4) The positive inotropic response to isoprenaline amounted to ~100% of basal TT in circular EHTs compared with only ~15% to 30% in the planar lattices. This better resembles the magnitude of the isoprenaline effect in intact rat preparations, for which we have reported an isoprenaline-induced increase in TT by 114% to 145% (EC₅₀ 0.11 μmol/L) under the same conditions, albeit at a calcium concentration of 1.8 mmol/L. 

The reason for the high sensitivity to isoprenaline in EHTs (EC₅₀ 2.8 nmol/L) remains unclear and parallels the previously observed leftward shift of the calcium response curve in the EHT model (EC₅₀ 0.46 mmol/L versus 3.1 mmol/L in adult rat papillary muscles). 

(5) EHTs are suitable for the electrophysiological investigations usually performed on isolated multicellular cardiac preparations, eg, papillary muscles. Six intracellular recordings on EHTs revealed stable resting membrane potentials and action potentials similar to those found in ventricular myocytes from young rats. 

(6) Cardiac myocytes in EHTs exhibited several morphological features of terminal differentiation: (a) densely packed and highly organized sarcomeres; (b) an adult cardiac myocyte–like volume ratio of

![Figure 8. Contractile response to calcium and isoprenaline. Top left, Effect of calcium (n=15, 7 independent cell preparations) was evaluated by cumulatively increasing calcium from 0.4 to 2.8 mmol/L (calculated EC₅₀ 0.46±0.06 mmol/L). Top right and bottom left, Effect of isoprenaline (n=12, 6 independent cell preparations) was tested at 0.4 mmol/L calcium by cumulatively increasing isoprenaline from 0.1 to 1000 nmol/L (EC₅₀ 2.8±0.6 nmol/L). Bottom right, Additionally, isoprenaline lowered RT. *P<0.05 indicates significant differences (by repeated-measures ANOVA with post hoc Bonferroni test) to force at 0.4 mmol/L calcium (top left panel) or predrug value (top right and bottom left and right panels).](http://circres.ahajournals.org/)

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myofilaments: mitochondria:nucleus of 45:24:9, with the remaining 23% consisting mainly of SR and cytosol, which compares favorably with published data on adult cardiac myocytes of 47:36:2, with the remaining consisting of 3.5% SR and 11.5% cytosol16; (c) all types of normal intercellular connective structures, such as adherens junctions, desmosomes, and gap junctions; (d) T tubules, SR vesicles, and T-tubule–SR junctions in the form of dyads; and (e) a well-developed basement membrane surrounding cardiac myocytes. It is important to note that some of the features observed in EHTs, especially the T-tubule–SR junctions, were found to be absent in the newborn rat heart17 and in monolayer cultures of cardiac myocytes.23 In addition, during the cell isolation procedure, cardiac myocytes lose or disassemble much of their myofilament equipment and appear as rounded cells at the time that they are put into the medium-collagen-Matrigel mix. Therefore, it is remarkable that during 14 days of in vitro cultivation, they surpass the differentiation state of their source tissue. (7) The electron microscopic investigation also revealed that cardiac cells form not only a myocyte network but also a complex heart-like structure with multiple layers of nonmyocytes at the surface and endothelial cells forming primitive capillaries inside EHTs. Fibroblasts and macrophages were seen throughout the EHTs, suggesting that EHTs represent a spontaneously forming cardiac “organoid.” The conditions controlling this process or its functional consequences have not been investigated in the present study, but the present findings may open the possibility of using this system as a model for in vitro cardiac development. (8) Finally, for in vitro applications, technical aspects are also important. The ring system requires only simple casting forms that can be used infinitly and that allow the routine production of more precise and highly reproducible EHTs in large series (see contraction experiments Figure 8). It also opens the way for a multwell apparatus for drug screening or target validation. Such a device is presently under construction.

Tissue Engineering

The replacement of defective cardiac tissue by functioning myocardium offers an exciting option in cardiovascular medicine.24,25 Two principle strategies have been tested so far, mainly in the cryoinjury or in the myocardial infarction model after coronary ligation in mice and rats. One approach uses isolated cells,26–35 and the other uses in vitro–designed tissue equivalents.4,5 In most studies, the injection of cells into the scar tissue improved global heart function. Surprisingly, the effect appeared to be independent of cell origin, because positive results were reported from fetal or neonatal cardiac myocytes, fibroblasts, endothelial cells, smooth muscle cells, skeletal myoblasts, and pluripotent stem cells.26–33 The concept of expanding autologous skeletal myoblasts ex vivo and injecting them into the postinfarction scar during coronary artery bypass grafting has already been transferred to humans, and the first results are promising.34 Despite survival and differentiation of implanted cells, mechanical and electrical cell-cell contacts between graft and host, a chief requirement for synchronous contractions, were only rarely observed in carefully designed studies.28,35 and accordingly, the proof of direct participation of the grafted material in overall cardiac contraction is lacking. Formation of scar tissue inhibiting contact between grafted cells and host tissue appears to account for this problem, at least in part.28,35 The most recently successful implantation of pluripotent stem cells into the infarction scar has been reported in mice.29 The exciting aspect of that report was that the stem cells acquired, at least in part, a cardiac phenotype, demonstrating the potential of an autologous adult stem cell approach.

An alternative approach to cell-grafting procedures is tissue replacement with in vitro–designed cardiac constructs. For in vitro tissue construction, several scaffold proteins and synthetically produced polymers have been tested, including collagen, gelatin, alginate, and polyglycolic acid.1–5 There are some principal problems of this approach: (1) Scaffold materials often exhibit an intrinsic stiffness that may compromise diastolic function. (2) Biodegradation of the scaffold materials remains incomplete, adding to the potential problems with diastolic function. (3) Size limitation of engineered constructs exists that are due to a lack of metabolic or oxygen supply in the core of 3D constructs.3,5,36 Li et al36 reported that cardiac myocytes seeded on or in gelatin meshes formed a 300-μm-thick cell layer only on the outside. Bursac et al36 observed that cardiac myocytes seeded on polymer scaffolds would form cell layers of 50 to 70 μm. A homogeneous cell distribution within the constructs was not achieved by either group. Core ischemia is well known in papillary muscles with diameters >100 μm.37,38 In rat hearts, the intercapillary distance is 17 to 19 μm.39 EHTs have some principal advantages and share some of these problems. In our view, advantages are the clearly longitudinally oriented, well-coupled network of muscle bundles, the remarkable degree of differentiation, a cardiac tissue–like contractile function including very low RT, and the organoid nature of the construct with a surface lining consisting of nonmyocytes and capillaries. These features should prove to be advantageous for survival, vascularization, and synchronous beating with the host myocardium. In addition, core ischemia is unlikely because the compact muscle bundles with a diameter of 30 to 100 μm (Figure 2a) were found throughout the EHTs without preferential formation at the outer layers. This indicates that the collagen matrix at the concentration used in the present study does not represent a significant diffusion barrier or, alternatively, is rapidly degraded. Yet, important limitations remain: (1) The cardiac tissue–like network in EHTs is (with the exception of the compact strands, Figure 2a) generally much less compact than that in native tissues (Figures 3 and 4), explaining why contractile force is, in absolute terms, ~10-fold less than that in comparable intact cardiac preparations. Very thin cardiac muscle preparations develop maximal twitch tension of ~20 mN/mm² in ferrets, rats, cats, rabbits, and humans.20 In contrast, maximal forces in EHTs amounted to 2 mN/EHT, ie, 2 mN/mm². (2) The degree of cardiac differentiation, despite being superior to 2D cultures (eg, T tubules and SR junctions), is clearly less than that in intact adult myocardium (eg, no mature M bands). (3) The compact muscle–like strands (Figure 2a) did not exceed 30 to 100 μm in diameter, which is in line with theoretical considerations and published data. Possibly, optimized culture conditions (growth factors, higher Po2, and culture in rotating flasks) could allow for thicker and more compact EHTs. (4) Finally and most important, it is unknown at present whether EHTs indeed can
serve as a tissue equivalent for replacement therapy and have advantages over cell-grafting approaches. These questions are currently under investigation.40

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Tissue Engineering of a Differentiated Cardiac Muscle Construct

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Material and Methods

Neonatal Rat Cardiac Myocytes

Rat cardiac myocytes were isolated from 1-3 day old neonatal Wistar rats (Friedrich-Alexander University Erlangen-Nuremberg breed, from Charles River, Bad Kisslegg, Germany) as described previously. To reduce non-myocyte contamination cells were preplated with serum-containing medium (DMEM, 10% fetal calf serum, 2 mmol/l L-glutamine) on Falcon® series 3000 dishes for 60 min. Cell yield per animal was 2-3x10⁶ viable cells as judged by trypan blue exclusion.

Engineered Heart Tissue

A typical reconstitution mixture for 4 EHTs is shown in Supplement Table 1. The optimal reconstitution mix has been the result of former extensive testing the effect of different original cell number (1-10x10⁶ cells per EHT) as well as the concentrations of collagen I (0.4-0.85 mg/EHT = 0.45-0.95 mg/ml), Matrigel® (0, 5, 10, 15%), and serum supplements (+/-chick
embryo extract, serum reduction at day 7) on tissue formation and force development. Part of these culture conditions were studied in the original lattice model,\(^1\) part in the ring model.\(^2\) An increase in cell number between \(1 \times 10^6\) and \(2.5 \times 10^6\) cells per EHT increased contractile force, further increases did not improve function, but led to faster condensation of the cell/matrix mix. At \(10 \times 10^6\) cells per EHT, EHTs disrupted before force measurement (unpublished data). Collagen I was found optimal at 0.5-0.8 mg/EHT, with less collagen resulting in unstable and only weakly contracting EHTs and more collagen yielding EHTs that were rigid and developed less active force. 0.8 mg were chosen for mechanical stability and easier handling. Matrigel was essential for any tissue development and improved force between 5 and 15%. 10% was chosen as a compromise between function and costs. Matrigel is a complex mixture of extracellular matrix proteins and growth factors, and we have not yet identified the component(s) responsible for the permissive effect. However, a soluble factor appears to play an important role.\(^1\) Chick embryo extract could be omitted on day 7 without significant effect on force development (earlier time points were not tested). In contrast, even small reductions in horse serum concentration (to 3%) starting on day 7, 8 or 9 of culture prevented condensation of the cell/matrix mix and almost completely abolished contractile function when measured at day 14.\(^2\) A more selective approach to investigate the role of culture medium supplements on tissue reconstitution and function has been employed by EHT culture in the presence of growth factors as an additional medium supplement-on top of serum-containing culture medium. By doing so we could show that certain growth factors can improve tissue reconstitution and function (Supplement Table 2).

**Casting Molds**

Circular casting molds (diameter: 16 mm, depth: 5 mm) were prepared in glass culture dishes (diameter: 50 mm). Two silicone tubes were glued to the surface of the culture dish to hold reversible teflon discs (diameter: 16 mm, depth: 5 mm) that served as spacers while liquid silicone glue was poured into the culture dish. After hardening of the silicone glue the teflon spacers were removed (Fig. 1a). To cast EHTs, a removable teflon cylinder (diameter: 8 mm) was placed over each silicone tubing to yield ring-shaped wells (750 mm\(^3\)). The size of a
casting mold was sufficient to hold the viscous reconstitution mixture (900 μl) in place (Fig. 1b; video sequence). Casting molds could be reused infinitely after sterilization.

**Force Measurement**

After 30 min equilibration without pacing, EHTs were electrically stimulated with rectangular pulses (2 Hz, 5 ms, 80-100 mA). Preload was adjusted to Lmax, i.e. the length were EHTs developed maximal active force. Inotropic and lusitropic responses to cumulative concentrations of calcium (0.4-2.8 mmol/l) and isoprenaline (0.1-1000 nmol/l) were measured. Twitch tension (TT), resting tension (RT), contraction duration (T1: time from 10% to peak force development), and relaxation duration (T2: time to 90% relaxation) were evaluated by BMON software (Ingenieurbüro Jäckel, Hanau, Germany).

**Light Microscopy**

EHTs were fixed in 4% formaldehyde/1% methanol in phosphate buffered saline (PBS), pH 7.4, containing 1 mmol/l CaCl₂ and 30 mmol/l 2,3-butanedione monoxime (BDM) overnight at 4 °C. After overnight wash in PBS, EHTs were embedded in 2% agarose blocks. Dehydration in graded concentrations of isopropanol, paraffin infiltration, and paraffin embedding were done according to standard procedures. Horizontal and cross sections (4 μm) were stained with hematoxylin & eosin (H&E) and photographed with a digital camera (Coolpix 950, Nikon) on a Zeiss Axioplan microscope.

**Confocal Laser Scanning Microscopy**

EHTs were fixed and embedded in agarose as described above or used as whole mount samples after fixation. Sections (100 μm) of agarose embedded EHTs were prepared with a vibratome slicer (Campden Instruments, UK). Vibratome and whole mount samples were blocked and permeabilized at 4 °C overnight in tris-buffered saline (TBS), pH 7.4 containing
10% fetal calf serum, 1% bovine serum albumine, 0.5% Triton-X 100, and 0.05% thimerosal. After two washes in TBS sections were incubated with primary antibodies (Supplement Table 3) to detect proteins preferentially expressed in cardiac myocytes (α-sarcomeric actin, α-sarcomeric actin, myomesin), smooth muscle cells (α-smooth muscle actin), fibroblasts (prolyl-4-hydroxylase), endothelial cells (CD31), and macrophages (ED2) at 4 °C for 48 h. Antibodies to detect myomesin (clone B-6) were a generous gift from M.C. Schaub and J.C. Perriard, Zürich, Switzerland. After an overnight wash in TBS sections were incubated with FITC-labeled anti-mouse IgG (1:256; Sigma) or IgM (1:128; Sigma) antibodies at room temperature for 3 h. After repeated washes sections were postfixed with 4% formalin/1% methanol (15 min at room temperature). Filamentous actin was labeled by incubation of samples with phalloidin-toxin coupled to TRITC (1 μmol/l, Sigma) for 1 h at RT. Sections were mounted in mowiol 4-88 (Calbiochem) with 2.5% diazabicyclo[2.2.2]octane (DABCO; Sigma). Confocal imaging was performed with a Zeiss LSM 5 Pascal system using a Zeiss Axiovert microscope. Extended focus imaging was achieved by superposition of 20 optical slices (4 μm; z-axis: 76 μm) and presented in black & white (Fig. 4a).

**Transmission Electron Microscopy**

EHTs were fixed in 2.5% glutaraldehyde in PBS, pH 7.4 containing 1 mmol/l CaCl₂ and 30 mmol/l BDM overnight at 4 °C. After overnight wash in PBS, EHTs were post fixed in osmiumtetroxyd/PBS (1:1) for 2 h at RT. Following an overnight wash in PBS at 4 °C samples were dehydrated, epon infiltrated, and epon embedded according to standard protocols. Semithin sections (1 μm) were stained with toluidin blue and diameter of cross sections (n=16) was measured. For TEM, ultrathin sections (50 nm) were cut (Ultracut UCT, Leica), contrasted with uranyl acetate and lead citrate, and examined with a Zeiss Leo 906
EM system. For TEM analyses series of longitudinal and cross sections were evaluated. Cells were identified by observation of cell species specific characteristics (Supplement Table 4).

**Rat Heart Tissue**

Cardiac myocyte morphology within EHT was compared to native myocardium from newborn (0. dpp), neonatal (6. dpp), and adult (300 g) Wistar rats. Hearts were excised rapidly, rinsed in PBS, and fixed before paraffin embedding as described above. Paraffin sections of heart tissue and EHT were prepared, stained with H&E, and investigated in parallel as described above.

**Electrophysiological Studies**

Intracellular action potentials were recorded as described previously with minor modifications. Briefly, circular EHTs were cut into quarters and one preparation was superfused with Tyrode’s solution (2 ml/min, 36°C; mmol/L: NaCl 126.7, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.05, NaHCO₃ 22, NaH₂PO₄ 0.42, glucose 5.6; pH 7.4). After equilibration for at least 60 min the EHT was electrically stimulated at 1 Hz with a current 5% over threshold. Action potentials were recorded with conventional microelectrodes of 10-25 MΩ tip resistance when filled with 2.5 M KCl. The recorded signals were amplified (KS-700, WPI, Berlin, Germany), displayed on an oscilloscope, and stored online. All data acquisition and analysis was carried out with an ISO-2 system (MFK, Niedernhausen, Germany).

**Statistics**

Data were calculated as arithmetic means±SEM and analysed using a repeated measures ANOVA followed by a Bonferroni post hoc test. A P-value of less than 0.05 was considered significant. "n" indicates the number of independent EHTs or cells investigated.

Morphometrical analysis was performed with NIH Image or Zeiss LSM software.
Legend for Supplementary Online Figure

Supplement Figure 1. Intracellular action potentials. Original intracellular recording of a representative EHT action potential at 36°C. * indicates the stimulus artifact. dV/dt max: maximum upstroke velocity; APA: action potential amplitude; APD: action potential duration at 20, 50, and 90% repolarization; RMP: resting membrane potential

Legends for Supplementary Online Tables

Supplement Table 1. Reconstitution mixture to yield 4 EHTs. Collagen type I (4.2 mg/ml in 0.1% acetic acid) prepared from rat tails was mixed with an appropriate volume (1:1) of concentrated culture medium (2xDMEM, 20% horse serum, 4% chick embryo extract, 200 U/ml penicillin, 200 μg/ml streptomycin) to yield basal medium and serum concentrations in the reconstitution mixture. Acidic pH was neutralized (pH ~7.4) by titration of 0.1 N NaOH. Supplementation with 10% basement membrane protein mixture has been shown to be mandatory for reconstitution of neonatal rat cardiac myocytes to EHT.¹ Finally, 11x10⁵ freshly isolated cardiac myocytes in a defined volume of culture medium (1722 μl; DMEM, 10% horse serum, 2% chick embryo extract, 100 U/ml penicillin, 100 μg/ml streptomycin) were added to yield 4 ml of reconstitution mixture. 900 μl of the well mixed reconstitution mixture were pipetted into circular casting molds to yield EHTs with 0.8 mg collagen and 2.5x10⁶ cells.

Supplement Table 2. Functional consequences of longterm stimulation with growth factors. Growth factors (IGF-1: Insulin-like growth factor-1; EGF: Epidermal growth factor; FGF-2: Fibroblast growth factor-2; ITS: Insulin/Transferrin/Selen; ET-1: Endothelin-1; TGFβ1: Transforming growth factor β1; CT-1: Cardiotrophin-1) were added to the culture
medium (DMEM, 10% horse serum, 2% chick embryo extract) and were renewed with every medium change (first 7 culture days every second day, thereafter daily). After 14 days in culture under described conditions isometric force was measured under field stimulation (2 Hz) in standard organ baths. * Indicates a significant difference (P<0.05, unpaired Student’s t-test) from mean control values. Note that absolute force (TT: twitch tension) of EHTs used for this study was slightly lower than observed in EHTs used for basal characterization (0.53±0.09 mN vs. 0.75±0.11 mN). However, inotropic responses to maximal calcium (1.6 mmol/l) were comparable (mN±SEM: 0.21±0.04 to 0.53±0.09 vs. 0.34±0.06 to 0.75±0.11; Fig. 8a). ET-1 and ITS were purchased from Sigma. All other growth factors were purchased from PeproTech.

**Supplement Table 3. Antibodies used for CLSM.** Antibodies were chosen to investigate distribution of cardiac myocytes (α-sarcomeric actinin, α-sarcomeric actin, myomesin), smooth muscle cells (α-smooth muscle actin), fibroblasts (prolyl-4-hydroxylase), endothelial cells (CD31), and macrophages (ED2) within EHTs. By incubation of vibratome sections and whole mount samples with secondary antibodies only (negative control) no specific staining was observed. Note that even after intensive washing background level of FITC fluorescence was high due to unspecific staining of extracellular matrix proteins.

**Supplement Table 4. Identification of cell species by ultrastructural characteristics.** The table summarizes ultrastructural properties that were used to identify different cell types in EHT.
References for Online Supplement


Video Sequence

Demonstration of (1) the casting procedure, (2) EHT transfer and culture in stretch device - note that this series shows stretching at a low frequency of 1 Hz, (3) spontaneously contracting EHT (low power microscopic view and macroscopic view), and (4) isometric force measurement in a standard organ bath.
Supplement Figure 1.

<table>
<thead>
<tr>
<th></th>
<th>mean±SEM</th>
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<tr>
<td>dV/dtmax (V/s)</td>
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<tr>
<td>APA (mV)</td>
<td>109±2</td>
<td>6</td>
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<tr>
<td>APD20 (ms)</td>
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<td>APD50 (ms)</td>
<td>87±4</td>
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<td>APD90 (ms)</td>
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<tr>
<td>RMP (mV)</td>
<td>-73±2</td>
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Supplement Table 1.

<table>
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<tr>
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<th>Mastermix: 4 EHTs</th>
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<tr>
<td>Collagen type I</td>
<td>847 µl (0.8 mg)</td>
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<tr>
<td>Concentrated culture medium</td>
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<tr>
<td>NaOH (0.1 N)</td>
<td>184 µl</td>
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<tr>
<td>Basement membrane protein mixture</td>
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<tr>
<td>Freshly isolated cells</td>
<td>1722 µl (11x10^6)</td>
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| Volume                         | 4000 µl           |
### Supplement Table 2.

<table>
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<tr>
<th>Growth factor</th>
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<tr>
<td>IGF-1</td>
<td>20 ng/ml</td>
<td>0.87±0.12 mN</td>
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<td>EGF</td>
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<td>0.80±0.09 mN</td>
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<td>FGF-2</td>
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<td>ITS</td>
<td>10 µg/ml Insulin</td>
<td>0.99±0.24 mN</td>
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<td></td>
<td>5.5 µg/ml Transferrin</td>
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<tr>
<td></td>
<td>5 ng/ml Selen</td>
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<td>ET-1</td>
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<td>CT-1</td>
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<td>TGFβ1</td>
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<td>Ctr.</td>
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<td>Antibody</td>
<td>Antigen Recognized</td>
<td>Dilution</td>
<td>Source</td>
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<tr>
<td>-----------------</td>
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<td>EA-53 (IgG1)</td>
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<td>1:1000</td>
<td>Sigma</td>
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<td>B-4 (IgG)</td>
<td>myomesin</td>
<td>fs!! strength</td>
<td>Dr. Perriard/Dr. Schaub, Zürich</td>
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<td>5C5 (IgM)</td>
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<td>α-smooth muscle actin</td>
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<td>ED2 (IgG)</td>
<td>ED2 antigen (macrophages)</td>
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<td>CD31 (PECAM)</td>
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<td>6-9H6 (IgG1)</td>
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Supplement Table 4.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ultrastructural properties</th>
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<tr>
<td>Cardiac myocytes</td>
<td>Sarcomeric organization of myofilaments, costameres, intercalated disks with fascia adherens, desmosomes and gap junctions, dyadic junctions, T-tubuli, basement membrane</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>Loose organization of myofilaments, focal adhesion plaques, caveolae, basement membrane</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Rich in endoplasmatic reticulum, no basement membrane</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Formation of primitive capillaries, endo- and exocytotic vesicles, tight junctions, basement membrane</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Lysosomes, pseudopodia, no basement membrane</td>
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