Development of a Drug Screening Platform Based on Engineered Heart Tissue

Arne Hansen,* Alexandra Eder,* Marlene Bönstrup, Marianne Flato, Marco Mewe, Sebastian Schaaf, Bülent Aksehirlioglu, Alexander Schwörer, June Uebeler, Thomas Eschenhagen

Rationale: Tissue engineering may provide advanced in vitro models for drug testing and, in combination with recent induced pluripotent stem cell technology, disease modeling, but available techniques are unsuitable for higher throughput.

Objective: Here, we present a new miniaturized and automated method based on engineered heart tissue (EHT).

Methods and Results: Neonatal rat heart cells are mixed with fibrinogen/Matrigel plus thrombin and pipetted into rectangular casting molds in which two flexible silicone posts are positioned from above. Contractile activity is monitored video-optically by a camera and evaluated by a custom-made software program. Fibrin-based mini-EHTs (FBMEs) (150 µL, 600,000 cells) were transferred from molds to a standard 24-well plate two hours after casting. Over time FBMEs condensed from a 12×3×3 mm gel to a muscle strip of 8 mm length and, depending on conditions, 0.2 to 1.3 mm diameter. After 8 to 10 days, FBMEs started to rhythmically deflect the posts. Post properties and the extent of post deflection allowed calculation of rate, force (0.1 to 0.3 mN), and kinetics which was validated in organ baths experiments. FBMEs exhibited a well-developed, longitudinally aligned actin-positive cardiac muscle network and lectin-positive vascular structures interspersed homogeneously throughout the construct. Analysis of a large series of FBME (n=192) revealed high yield and reproducibility and stability for weeks. Chromanol, quinidine, and erythromycin exerted concentration-dependent increases in relaxation time, doxorubicin decreases in contractile force.

Conclusions: We developed a simple technique to construct large series of EHT and automatically evaluate contractile activity. The method shall be useful for drug screening and disease modeling. (*Circ Res. 2010;107:35-44.)

Key Words: cardiac tissue engineering ■ drug screening ■ predictive toxicology ■ automation

Over the past decade, techniques have been developed to generate cardiac tissue-like 3D constructs in vitro.1 Tissue engineered myocardial constructs may serve as means for cell-based cardiac repair and as improved in vitro models for predictive toxicology and target validation, taking advantage of a more physiological cellular environment. Recent cardiac tissue engineering approaches include the use of solid, preformed scaffolds,2–7 matrix-free generation of tissues from stackable cell sheets,8 or the generation of constructs in preformed casting molds using hydrogels such as collagen I, Matrigel, fibronectin, or fibrin.9–13 The principal usefulness of these techniques for cardiac repair14 and in vitro testing15 has been demonstrated.

Using circular casting molds and collagen I plus Matrigel to generate engineered heart tissue (EHT) from neonatal rat heart cells we have shown that EHT develop a high degree of cellular differentiation, longitudinal orientation, intercellular coupling and force generation.10 Tissue formation and force generation of EHT were improved by phasic16 or auxotonic stretch, increased oxygen concentration and supplementation with insulin.14 Others demonstrated beneficial effects of electric stimulation.6 Recent methods to generate cardiac myocytes from human embryonic stem cells17 or induced pluripotent stem cells18 have opened the realistic and exciting perspective to use these techniques for the validation of hypotheses and testing drugs in healthy and diseased human heart muscles.19

Yet, the existing techniques are either not suitable for this purpose (stacked cell sheet technique) or exhibit drawbacks that limit their usefulness. For example, the existing methods require extensive handling which precludes routine handling of large series and gives rise to experimental variability.

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From the Department of Experimental and Clinical Pharmacology and Toxicology (A.H., A.E., M.B., M.F., S.S., B.A., J.U., T.E.), Institute of Pharmacology for Pharmacists (M.M.), and Institute of Physiology (A.S.), Cardiovascular Research Center, University Medical Center Hamburg-Eppendorf, Germany.

*Both authors contributed equally to this work.

Correspondence to Thomas Eschenhagen, Institute of Experimental and Clinical Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. E-mail t.eschenhagen@uke.de

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Moreover, the collagen-based ring EHT technique requires relatively high numbers of cells and is difficult to miniaturize.

The present study aimed at miniaturizing the EHT format, multiwell-testing, and automated evaluation. One of the key factors was exchanging collagen I with fibrin(ogen). Fibrinogen is a 340-kDa glycoprotein, which is present in blood plasma at concentrations of 1.5 to 4 g/L and can be readily purified from different species. Characteristic mechanical properties of fibrin polymer are its nonlinear elasticity resulting in higher elastic modulus under shear stress and its softness in comparison to other filamentous biopolymers.20  
The degree of softness and elasticity depends on several different factors like fibrinogen concentration and introduction of bonds by plasma transglutaminase (factor XIII).20 In contrast to other extracellular matrices, the in vitro polymerization of fibrin results in a gel which is very similar to in vivo fibrin polymer. The gel is fully biodegradable by fibrinolytic enzyme plasmin. The availability of fibrinogen from autologous sources, its degradability, the versatile mechanical characteristics and the ability to covalently bind growth factors21 make fibrin an interesting compound for tissue engineering applications.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Manufacturing Sylgard Posts and Teflon Spacers

Sylgard 184 silicone elastomer (Dow Corning) was used to produce silicone post racks in custom-made Teflon casting molds. The 2-component Sylgard 184 elastomer was handled according to manufactures instructions and degassed under vacuum conditions before casting. The racks contained 4 pairs of posts and had the following geometry (Figure 1A): length/width of rack: 79×18.5 mm, length of posts 12 mm, diameter 1 mm, plate diameter 2 mm, distance (center–center) 8.5 mm. They are currently autoclaved and reused. Teflon spacers for producing the casting molds (Figure 1B) had the following geometry: length 12 mm, width 3 mm, height 13.5 mm.

Generation of Fibrin-Based Mini-EHTs

To generate fibrin-based mini-EHTs (FBMEs), a reconstitution mixture was prepared on ice as follows (final concentration): 4.1×10^6 cells/mL, 5 mg/mL bovine fibrinogen (stock solution: 200 mg/mL plus aprotinin 0.5 μg/mg fibrinogen in NaCl 0.9%, Sigma F4753), 100 μL/mL Matrigel (BD Bioscience 356235). DMEM (2×) was added to match the volumes of fibrinogen and thrombin stock to ensure isotonic conditions. Casting molds were prepared by placing the Teflon spacer in 24-well culture dishes and adding 1.6 mL 2% agarose in PBS (Invitrogen 15510-027) per well. After agarose solidification the spacer were removed (yielding a casting mold of 12×3×4 mm) and silicon post racks were placed onto the dishes with pairs of posts reaching into each casting mold. For each FBME 145 μL reconstitution mix was mixed briefly with 4.5 μL thrombin (100 U/mL, Sigma Aldrich T7513) and pipetted into the agarose slot.

For fibrinogen polymerization the constructs were placed in a 37°C, 7% CO₂ humidified cell culture incubator for 2 hours. Cell culture medium (300 μL) was then added per well to ease removal of the constructs from agarose casting molds. The racks were transferred to new 24-well cell culture dishes. Constructs were maintained in 37°C, 7% CO₂ humidified cell culture incubator. Media was changed on Mondays, Wednesdays and Fridays. EHT medium consisted of DMEM (Biochrom F0415), 10% horse serum ( Gibco 26050), 2% chick embryo extract, 1% penicillin/streptomycin (Gibco 15140), insulin (10 μg/mL, Sigma-Aldrich I9278), tranexamic acid (400 μmol/L, Sigma-Aldrich 857653), and aprotinin (33 μg/mL, Sigma Aldrich A1153).

Video Optical Analysis

The setup for video optical recording consisted of a cell incubator unit with control of CO₂, humidity and temperature, and a glass roof for monitoring purposes. Light diodes were positioned underneath the cell culture dish. A Basler camera (Type A 602f-2) placed above the cell culture unit was positioned in XYZ direction (IAI Corporation) in a PC-controlled manner. Video optical recording and light exposure were synchronized to minimize heating of cell culture
medium by light. Figure 2A illustrates the experimental setup. Video optical analysis was performed with a customized software package by Consulting Team Machine Vision (Pforzheim, Germany). The software is based on figure recognition of the contracting muscle strip at top and bottom ends in a fully automated manner. The distance between the ends of the muscle strip is determined during contractions and recorded over time. Based on post geometry (diameter, length), elastic modulus of Sylgard 184 (2.6 kPa) and delta of post distance (post deflection) force was calculated according to a recently published equation. Recorded contractions are identified by peak criteria (threshold value, minimum relaxation). Based on identified contractions, values for frequency, average force, fractional shortening, contraction – and relaxation time (T1, T2, respectively) were calculated. Records of experiments are automatically generated with two levels of quality control: pictures are taken at the beginning and the end of each measurement and blue squares indicate the positions on both ends of the muscle stripe (Figure 2C). Contractions are recorded as force development over time and green squares in the graph indicate the identified peaks (Figure 2D and Online Movie). The effort to analyze contractility with this setup is limited to defining the $x$, $y$, $z$ coordinates of the camera for each FBME.

Results

General Aspects of the New Technique

We present a new technique to generate FBMEs from neonatal rat heart cells. The technique is based on the preparation of a single cell suspension in a fibrinogen containing mastermix. Strip format casting molds are prepared from agarose in 24-well cell culture dishes and pairs of silicone posts are positioned in each mold. After addition of thrombin the cell preparation is transferred to the molds. Fibrin polymerization leads to the development of a hydrogel which is fixed to the silicone posts at both ends. 2 hours later the constructs are transferred to new cell culture dishes and maintained under cell culture conditions. Figure 1C illustrates this procedure.

Over time of cultivation, the cells spread inside the matrix, form cell–cell contacts and remodel the hydrogel. This process leads to marked condensation of the construct and imposes mechanical load on the cells and bends the posts toward each other. The final length of FBMEs thus amounted on average to 6.3 mm compared to 8.5 mm on day 0 (see Results, force of contraction). This leads to “individually optimized preload” of each construct and straight force lines within the FBMEs. This simplified procedure allows the routine preparation of 48 to 72 FBMEs per cell preparation (30 neonatal rat hearts). Figure 2B illustrates a typical 24-well plate. The absence of protease inhibitor aprotinin leads to a rapid degradation of the fibrin hydrogel (not shown). Addition of aprotinin at a concentration of 33 $\mu$g/mL markedly slowed this process, but did not entirely stop it. Stability could be enhanced by the addition of the
protease inhibitor tranexamic acid which allowed cultivation of FBMEs for extended periods of time (data not shown). The addition of tranexamic acid markedly slowed the reduction in diameter (final width 1.3 to 1.4 mm [Figures 2C and 6D] instead of 0.2 to 1 mm in its absence [Figure 3A]).

Morphology and Beating Activity
Directly after casting, FBMEs appeared as soft and flexible rectangular blocks of gel between two silicone posts (12 x 3 x 3 mm). The gel contained evenly distributed amorphous round heart cells (Online Figure I, A). Within days, cells elongated, aligned along force lines, and started to beat as single cells at day 4 to 5. Degradation and remodeling of extracellular matrix led to a marked reduction of construct size (final diameter 1 to 2 mm with and 0.2 to 1 mm without tranexamic acid), increased cellular density, and formation of small groups of cardiac myocytes (Online Figure I, B through E) and, at day 7 to 9, to coherent beating. By day 10, force of contraction was sufficient to rhythmically deflect the posts. Measurements were routinely done between day 14 to 16. At this point of development cardiomyocytes appear as approximately 100 to 200 μm long spindle-shaped cells with maximum diameter of 10 to 20 μm (Online Figure I, F).

Hematoxylin/eosin-stained paraffin sections of mature FBMEs (day 15) showed a dense, well-developed, strictly longitudinally aligned network of heart muscle tissue (Figure 3A). Importantly, cells were homogeneously distributed throughout the diameter of the FBME without a reduction in cellular density in center areas. These data suggest a high degree of cell survival and the absence of nutrient and oxygen shortage. Immunofluorescent analyses of whole-mount FBMEs showed a dense network of α-actinin-positive, longitudinally aligned, elongated cardiac myocytes with well-developed sarcomeric structure reaching into the periphery of the cytoplasm. Cardiomyocytes were also characterized by connexin-43 positive gap junctions mostly on lateral parts of the cell membrane. In addition, lectin-positive endothelial cells formed a network of primitive tube-like structures interspersed between cardiac myocytes (Figure 3).

DNA/RNA Content, Histone H3 Phosphorylation, and Caspase-3
The morphological data were supported by measurements of DNA and RNA content. Although DNA content dropped by 20% within the first 3 days, it remained stable for at least 2 weeks thereafter. The amount of total RNA per FBME dropped by 50% within the first 3 days from 3.2 to 1.6 μg and remained at this level for at least two weeks (Online Figure II, A and B). Quantification of histone H3 phosphorylation as a marker of proliferative activity and caspase-3 activity as a marker of apoptosis (Online Figure II, C and D) revealed an overall low level of histone H3 phosphorylation that further decreased over time. Caspase-3 activity was high immediately after cell isolation and then quickly declined, reaching background levels around day 9. These data suggest that a fraction of cells died after cell isolation, accounting for the initial drop in DNA and RNA content but that proliferative and apoptotic activity remained low thereafter.

Cardiac Marker Gene Expression Over Time
To get insight into the degree of cardiac myocyte maturation over time of FBME culture, transcript concentrations of some
known markers of cardiomyocyte maturation (α-actinin, SR Ca$^{2+}$ ATPase [SERCA], α- and β-myosin heavy chain [α/β-MHC], Na$^+$/Ca$^{2+}$ exchanger, titin) were analyzed at different stages of FBME development, starting with the reconstitution mix (day 0), and compared with those in intact neonatal and adult rat heart (Figure 4). To exclude potential confounding effects by the initial decrease in myocyte number, values were normalized to the mRNA concentration of the cardiac myocyte-specific protein calsequestrin 2. The expression profile of the six markers was roughly superimposable. In phase 1 (day 0 to 6), ie, the time characterized morphologically by single cells spreading out and forming clusters, transcript concentrations remained relatively stable. In phase 2 (day 9 to 12) transcript levels exhibited a sharp increase and peak, coinciding with the time FBME generally started to rhythmically deflect the posts (Figure 4). To exclude potential confounding effects by the initial decrease in myocyte number, values were normalized to the mRNA concentration of the cardiac myocyte-specific protein calsequestrin 2. The expression profile of the six markers was roughly superimposable. In phase 1 (day 0 to 6), ie, the time characterized morphologically by single cells spreading out and forming clusters, transcript concentrations remained relatively stable. In phase 2 (day 9 to 12) transcript levels exhibited a sharp increase and peak, coinciding with the time FBME generally started to rhythmically deflect the posts. In phase 3 (day 12 to 15), ie, the time characterized by coherent beating of FBMEs, transcript levels decreased and in the case of some markers (α-actinin, SERCA, α-MHC) conformed to expression levels of adult myocardium. In contrast, β-MHC, titin and Na$^+$/Ca$^{2+}$ exchanger mRNAs remained severalfold higher than in adult rat heart, potentially indicating higher remodeling activity.

Nonquantitative Ion Channel Expression Profile
To get initial information about the usefulness of the FBME system for the evaluation of proarrhythmic effects of drugs transcript concentrations of 23 ion channels subunits (7 calcium channels, 6 sodium channels, 10 potassium channels) known to be important in mammalian cardiac myocyte electrophysiology were amplified from FBME total RNA and compared to profiles from human non failing hearts (35 PCR cycles). As shown in Online Figure III, 22 of 23 transcripts were amplified from both samples. CacnAII was undetectable.

Action Potentials
Action potentials were recorded from 12 different FBMEs, both under electrically stimulated (n=6) and spontaneous (n=6) beating (Figure 5). Resting membrane potentials amounted to about −79 mV. Average action potential duration at 80% repolarization of spontaneous and stimulated action potentials amounted to 143 milliseconds and 164 milliseconds, respectively. Spontaneous action potentials with a beating rate of more than 2 Hz showed pacemaker cell characteristics with spontaneous diastolic depolarization.

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**Figure 4.** Quantitative RT PCR of FBME in comparison to neonatal and adult rat heart. ΔΔCT values were generated by normalization to calsequestrin 2 (average CT values for normalization were as follows: d0: 20.8, d3: 20.4, d6: 20.6, d9: 21.3, d12: 21.6, d15: 20.5). Figures show relative expression compared to day 0. Each bar represents results from 12 measurements (4 biological replica×3 technical replica). *P<0.05 vs day 15 (Student t test). Bars show means±SEM.
Force of Contraction

Force development of FBMEs generally increased between day 9 and 15 of culture and then remained roughly stable. Depending on the cell preparation and cell culture supplements such as insulin the calculated force as measured by video-optical recording varied between 0.05 and 0.4 mN. To validate video-optical measurements, representative FBMEs were transferred to thermostatted organ baths to determine isometric force of contraction under electric stimulation (2 Hz). Silicone posts were cut from the rack with scissors and carefully inserted into little silicone tubes fixed to the force transducer on the one and a fixed holder on the other side. This procedure was prone to mechanical damage. Yet, the experiment demonstrated that FBMEs developed force and responded to increasing preload (Frank-Starling mechanism) and extracellular calcium concentration with an increase in force (Online Figure IV). Twitch tension under maximally effective calcium concentration and optimized preload (L_{max}) amounted to 0.9 mN, which compared to the 0.3 mN measured video-optically and in the absence of pacing and optimized preload.

To determine the robustness of the new assay 6 independent series of one to two 24-well plates each (total 192 FBMEs) were generated under standard conditions (cell culture supplementation with aprotinin, insulin, and tranexamic acid). Two FBMEs could not be transferred from the casting mold, 4 constructs were not recognized by the software and 17 constructs did not contract during one minute recording time at day 15. Thus, the total success rate was 89% (169/192; Figure 6). Average force per series (day 15) ranged between 0.11 and 0.22 mN (SD in a series 7.6%). Contraction time (T1 = time to peak) ranged between 66 and 81 milliseconds (series SD 41%), relaxation time (T2 = time from peak to 80% relaxation) between 67 and 88 milliseconds (series SD 25%), frequency between 162 and 20 beats (series SD 109%), construct diameter between 1.3 and 1.4 mm (series SD 9.9%) and length between 6.7 and 5.6 mm (series SD 16.7%). The considerably larger construct diameters in this experiment were attributable to the presence of tranexamic acid.

Proarrhythmic and Cardiotoxic Effects of Drugs

To test whether the new assay is suitable for the detection of drug effects we tested compounds with known repolarization-inhibitory and cardiotoxic effects. The experimental I_{Ks} blocker chromanol 293B and the clinically used drugs quinidine and erythromycin (both associated with torsades des pointes arrhythmias) induced a delay in T2 (relaxation time; Figure 7). The effect of chromanol was already seen at 1 µmol/L and reached >7-fold prolongation of T2 at 100 µmol/L. The well-known cardiotoxic drug doxorubicin exerted a time- and concentration-dependent decrease in force and led to complete arrest of contraction at 1 µmol/L after 3 days (Figure 8). Interestingly, at early time points after addition of lower concentrations (0.1 µmol/L), doxorubicin significantly increased force.

Discussion

We developed a new method to generate spontaneously beating 3D heart muscle constructs in vitro. This protocol is robust, reproducible, and simple and allows measurements in a 24-well format with limited numbers of cells. This technique implemented three principal variations compared to previously established EHT protocols. (1) The use of fibrinogen and thrombin instead of freshly neutralized collagen I allowed faster solidification of the hydrogel than gelation of collagen. Polymerization of the hydrogel occurred in a few minutes and allowed transfer of constructs from the casting molds to a new cell culture dish after two hours. Faster solidification was associated with more homogeneous and reproducible cell distribution in the initial gel. It also allowed using 50% higher cell concentrations (0.6 × 10^6/150 µL versus 2.5 × 10^6/900 µL), because adverse concentration of cells at the bottom was prevented. Advantages of fibrin for future applications are its availability from autologous sources for
transplantation purposes and the ability to covalently couple growth-promoting and angiogenic factors.\(^{21}\) (2) Changing the format from ring to strip allowed miniaturization (volume reduction from 900 to 150 \(\mu\)L) and automation in the 24-well format with minimal nonstandardized handling. (3) Video-optical evaluation of contractile force supersedes the manual transfer and measurement of EHTs under nonstandardized condition and allows simple repeated measurements of large series.

The experimental setup (rectangular casting molds and two flexible silicone posts) was inspired by a system developed by Vandenburgh et al for skeletal myocytes\(^{22}\) and was turned upside down to simplify handling and allow video-optical recording from above. In combination with fibrin it has

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**Figure 6. Reproducibility of the assay.** FBMEs were generated at 6 different time points (series 1 to 6) and spontaneous activity was recorded on day 15. Parameters of contractility (force [A], frequency [B], contraction time T1, relaxation time T2 [C]) and construct dimensions (D) were averaged and compared. Minimal and maximal values were used to test for significant differences and are indicated with \(\ast P<0.05\) (Student t test). Bars show means±SD. Analyzed FBMEs for each series were: series 1: \(n=21\); series 2: \(n=24\); series 3: \(n=20\); series 4: \(n=18\); series 5: \(n=39\); series 6: \(n=47\).

**Figure 7. Effect of repolarization inhibitors on FBME contraction (T1) and relaxation time (T2).** FBMEs were incubated with increasing concentrations of the indicated compounds and evaluated before application of drug (baseline) and after each concentration. Note the absence of effect of all compounds on T1 and the concentration-dependent increase in T2 with chromanol, quinidine, and erythromycin (at 1000 \(\mu\)mol/L erythromycin, FBMEs discontinued contractile activity). A typical alteration of contraction peak morphology with increasing concentrations of quinidine, chromanol, and erythromycin is shown in Online Figure V. \(\ast P<0.05\) (Student t test). Each spot represents 1 analyzed FBME.
several important features. (1) It is simple and uses standard 24-well cell culture plates. In fact, except for the silicone racks which, at this point, still need to be casted manually in Teflon molds, it does not need specific instrumentation and can be evaluated on a standard microscope or even by eye. (2) The method is robust and reproducible: 97% of FBMEs were analyzed by video optical recording on day 15. Eighty-nine percent showed spontaneous contractile activity at the moment of recording. (3) Cardiac tissue development in the FBMEs was excellent with a strictly longitudinally oriented, well-developed cardiac myocyte network forming a homogenous and dense heart muscle tissue. Three reasons likely contribute to this observation: First, fast polymerization of fibrin caused more even cell distribution throughout the gel and allowed us to use higher cell density. Second, cell survival appears very high in fibrin matrix. This was substantiated by the stability of total DNA content, showing only a minor drop between day 0 and 3, relatively stable RNA concentration (50% to 70% of initial value) and a decrease of initially high caspase-3 activity to essentially zero at day 9. Conversely, the proliferative activity in FBMEs as determined by the levels of histone H3 phosphorylation appears very low, comparable to 3H-thymidine incorporation data obtained in EHTs earlier. Stable mRNA concentrations of cardiac myocyte marker genes such as α-actinin, α-MHC, titin, calsequestrin and SERCA are also compatible with an overall relatively stable experimental system. Interestingly, the fetal gene marker β-MHC showed a severalfold decrease between day 0 and 6 and then, concomitantly, with the start of macroscopic contractions, a ≈5-fold increase to day 9. Although these alterations could indicate immaturity of cardiac gene expression in FBMEs, they could also indicate remodeling. The fact that SERCA and titin mRNAs showed increased concentrations over time rather argues for remodeling and maturation. Third, the silicone post method exposes the developing FBMEs to a directed mechanical load, which, in contrast to the motorized phasic stretch used for the ring-EHTs previously, is adapted to the individual construct. If the tonic, diastolic force of the heart muscle tissue is high, the FBMEs deflect the posts strongly and vice versa. It also allows the FBMEs to perform contractile work against the elastic posts, similar to the beating heart working against the afterload exposed by the circulation. We have previously shown that this kind of “auxotonic” load improves tissue development of EHTs.14

Important requisites toward a screening platform were miniaturization, reduced manual handling and automated readout. FBME generation requires 4.2-fold lower cell num-

Figure 8. Doxorubicin toxicity on FBMEs. FBMEs were incubated in the presence of doxorubicin (0.1 to 1000 nmol/L, starting at day 13 of culture), average forces were determined daily. Whereas doxorubicin at 0.1 μmol/L increased force after 24 hours, higher concentrations (1 μmol/L) led to a time-dependent reduction in force development. *P<0.05 (Student t test). Bars show means±SEM; number of evaluated (beating) constructs as indicated.
bers and additional reduction is possible. However, at this point, the 24-well format appears as a good compromise between miniaturization and ease of handling. The latter is reduced to transfer of entire silicone racks and much easier than the manual transfer of ring-EHTs from casting molds to motorized, static or auxotonic stretchers and into the organ baths for measurement. Video-optical recording of contractile activity turned out to be simple and robust. Parallel measurements of isometric force in organ baths revealed that the calculated forces roughly represent true values (0.3 versus 0.9 mN). The lower calculated values may be attributable to a systematic error but are more likely attributable to the absence of electric pacing and preload optimization. In absolute terms, forces of FBME are still lower than the 50 mN/mm² reached in intact heart muscle. However, if one assumes the true force to be 0.9 mN (organ bath) and the minimal diameter of the construct 0.2 mm (Figure 3), relative force amounts to up to 28.7 mN/mm² which approaches physiological values. Instrumental for the evaluation of quantitative data were the development of a software algorithm that automatically detects FBME edges and quantifies their movements over time, calculates force, frequency, times of contraction and relaxation and generates a report summarizing the results.

The experiments with proarrhythmic drugs and the known cardiotonic doxorubicin revealed principal suitability of the FBME system as a platform for drug screening. The known Iₖs blockers chromanol and quinidine exerted marked prolongation of relaxation time. The necessary concentration of 100 μmol/L appears high, but IC₅₀ values in H9c2 cells amounted to 8 and 10 μmol/L, respectively, and 100 μmol/L was necessary to fully block Iₖs. This may suggest that an almost full inhibition of Iₖs is required to see delayed relaxation in this system. Erythromycin, an antibiotic associated with torsades des pointes arrhythmia in men, Iₖr- but not Iₖs-inhibitory activity in dog²⁵ and action potential prolongation in rat ventricular myocytes (IC₅₀ 60 μmol/L²⁶), also prolonged relaxation at 100 μmol/L and above. Doxorubicin had stimulating effects at 0.1 μmol/L and early time points and completely stopped contractile activity at high concentrations and extended periods of incubation. One could speculate that the initial increase in force relates to the reactive oxygen species—generating effect of this compound, which then, at higher concentration and longer time, converts into toxicity. Yet, detailed analysis of the underlying mechanisms of both the proarrhythmic and cardiotonic drug effects was beyond the scope of this study and clearly requires further work.

The presented technique still has some limitations. (1) Manufacturing of silicone posts is done manually, leading to some variations in geometry and elastic properties and thereby confounding force calculations. Industrial fabrication is warranted. (2) The quality of rat heart cell preparation exhibits some variability. Whereas standard deviations inside each series of FBMEs were low, mean values for force and frequency varied between series by a factor of 2 and 8, respectively. This requires further investigations of parameters impacting on FBME development and the development of techniques to measure force under electric stimulation at fixed rate. (3) Spindle-shaped cardiomyocytes with a length/width ratio of ≈10 and a predominantly lateral orientation of connexin-43–positive gap junctions suggest that cardiomyocytes, despite functional, molecular, and morphological indices of maturation, do not acquire a truly adult phenotype during FBME development. The impact of these observations remains unclear.

However, the overall similarity of physiological characteristics between FBMEs and in vivo heart tissue is reassuring. In context with the observed prolongation of relaxation in the presence of proarrhythmic drugs, this study suggests that FBMEs might be useful for in vitro drug screening, predictive toxicology, and disease modeling.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

- Tissue engineering aims at generating 3D tissues outside of the body for 2 major applications: organ repair and advanced in vitro modeling of organ function.
- Several methods have been developed to generate engineered heart tissues from primary heart cells. However, their practical usefulness has been limited by lack of robustness and automation.

What New Information Does This Article Contribute?

- This article describes a technique to generate miniaturized 3D, force-generating engineered heart tissues from neonatal rat heart cells in a standard 24-well format. The main features are a high level of robustness and reproducibility, reduced manual handling, and automated video-optical analysis of contractility. The method is based on fibrin as a hydrogel matrix and self-assembly of the myocardial tissue between 2 flexible silicon posts that support tissue formation under directed mechanical strain. The constructs can be maintained under cell culture conditions for extended periods of time and subjected to repeated video-optical measurements of contractile force, frequency and contraction kinetics. We show that the method allows detection of force-decreasing effects of the cardiotoxic doxorubicin and relaxation-prolonging effects of the proarrhythmic compounds erythromycin, chromanol 293B, and quinidine. This study opens the realistic possibility to use engineered heart tissues for comprehensive drug screening and disease modeling purposes. The method might be particularly useful in combination with cardiomyocytes derived from human pluripotent stem cells.

- This article describes a new simple technique to generate miniaturized 3D force-generating engineered heart tissues from neonatal rat heart cells in a standard 24-well format. The main features are a high level of robustness and reproducibility, reduced manual handling, and automated video-optical analysis of contractility. The method is based on fibrin as a hydrogel matrix and self-assembly of the myocardial tissue between 2 flexible silicon posts that support tissue formation under directed mechanical strain. The constructs can be maintained under cell culture conditions for extended periods of time and subjected to repeated video-optical measurements of contractile force, frequency and contraction kinetics. We show that the method allows detection of force-decreasing effects of the cardiotoxic doxorubicin and relaxation-prolonging effects of the proarrhythmic compounds erythromycin, chromanol 293B, and quinidine. This study opens the realistic possibility to use engineered heart tissues for comprehensive drug screening and disease modeling purposes. The method might be particularly useful in combination with cardiomyocytes derived from human pluripotent stem cells.
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In the article that appears on page 35 of the July 9, 2010 issue, an author’s name is misspelled. The name should have appeared as Alexander P. Schwoerer.

This error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/107/1/35.full.

Reference:


DOI: 10.1161/RES.0b013e31823efb08
Supplement Material

Materials and Methods

Cell Isolation
Unpurified heart cells were isolated from neonatal Wistar rats (postnatal day 0 to 3) by a fractionated DNase/Trypsin digestion protocol as previously described (1). The resulting cell population was immediately subjected to FBME generation. Experimental procedures were reviewed and approved by Ethics Committee, University Hamburg.

Manufacturing Sylgard posts and teflon spacers
Sylgard 184 silicone elastomer (Dow Corning) was used to produce silicone post racks in custom-made Teflon casting molds. The two-component Sylgard 184 elastomer was handled according to manufactures instructions and degassed under vacuum conditions before casting. The racks contained 4 pairs of posts and had the following geometry (Figure 1A): length/width of rack: 79 x 18.5 mm, length of posts 12 mm, diameter 1 mm, plate diameter 2 mm, distance (center-center) 8.5 mm. Teflon spacers for producing the casting molds (Figure 1B) had the following geometry: length 12 mm, width 3 mm, height 13.5 mm.

Generation of FBMEs
To generate FBMEs, a reconstitution mix was prepared on ice as follows (final concentration): $4.1 \times 10^6$ cells/ml, 5 mg/ml bovine fibrinogen (stock solution: 200 mg/ml plus aprotinin 0.5 µg/mg fibrinogen in NaCl 0.9%, Sigma F4753), 100 µl/ml Matrigel (BD Bioscience 356235). 2xDMEM was added to match the volumes of fibrinogen and thrombin stock to ensure isotonic conditions. Casting molds were prepared by placing the teflon spacer in 24-well culture dishes and adding 1.6 ml 2% agarose in PBS (Invitrogen 15510-027) per well. After agarose solidification the spacer were removed (yielding a casting mold of 12x3x4 mm) and silicon post racks were placed onto the dishes with pairs of posts reaching into each casting mold. For each FBME 145 µl reconstitution mix was mixed briefly with 4.5 µl thrombin (100 U/ml, Sigma Aldrich T7513) and pipetted into the agarose slot.

For fibrinogen polymerisation the constructs were placed in a 37 ºC, 7% CO₂ humidified cell culture incubator for 2 hours. 300 µl of cell culture medium was then added per well to ease removal of the constructs from agarose casting molds. The racks were transferred to new 24-well cell culture dishes. Constructs were maintained in 37 ºC, 7% CO₂ humidified cell culture incubator. Media was changed on Mondays, Wednesdays and Fridays. EHT medium consisted of DMEM (Biochrom F0415), 10% horse serum (Gibco 26050), 2% chick embryo extract, 1% Penicillin/Streptomycin (Gibco 15140), insulin (10 µg/ml, Sigma-Aldrich I9278), tranexamic acid (400 µM, Sigma-Aldrich 857653) and aprotinin (33 µg/ml, Sigma Aldrich A1153).
Video optical analysis

The setup for video optical recording consisted of a cell incubator unit with control of CO₂, humidity and temperature, and a glass roof for monitoring purposes. Light diodes were positioned underneath the cell culture dish. A Basler camera (Type A 602f-2) placed above the cell culture unit was positioned in XYZ direction (IAI Corporation) in a PC-controlled manner. Video optical recording and light exposure were synchronized to minimize heating of cell culture medium by light. Figure 2A illustrates the experimental setup. Figure 2B shows a 24-well cell culture plate from above. Video optical analysis was performed with a customized software package by ctmv.de. The software is based on figure recognition of the contracting muscle strip at top and bottom ends in a fully automated manner. The distance between the ends of the muscle strip is determined during contractions and recorded over time. Based on post geometry, elastic modulus of Sylgard 184 (2.6 kPa) and delta of post distance (post deflection) force was calculated according to a recently published equation (3). Recorded contractions are identified by peak criteria (threshold value, minimum relaxation). Based on identified contractions, values for frequency, average force, fractional shortening, contraction – and relaxation time (T1, T2, respectively) were calculated. T1 and T2 were determined at 20% of peak maximum. Records of experiments are automatically generated with two levels of quality control: pictures are taken at the beginning and the end of each measurement and blue squares indicate the positions on both ends of the muscle stripe (Figure 2C). Contractions are recorded as force development over time and green squares in the graph indicate the identified peaks (Figure 2D, Online Movie). The effort to analyse contractility with this setup is limited to defining the x-, y-, z-coordinates of the camera for each FBME.

Histology

Formaldehyde-fixed FBMEs were sectioned or processed as whole mounts for light or confocal laser scanning microscopy as described previously (2). Hematoxylin and Eosin (H&E) staining was performed as described earlier (2). Confocal laser scanning microscopy was performed with a Zeiss LSM 510 META system.
Antibodies/lectin were used as follows: Bandeiraea simplicifolia lectin-TRITC 100 µg/ml 1:100 (Sigma L5264), α-actinin 1:800 (Sigma A7811), DRAQ5 1:1000 (Biostatus limited BOS-889-001-R050.), Alexa fluor® 488 1:800 (Goat anti mouse 2 mg/ml, Molecular Probes A 11017), Alexa fluor® 546 1:800 (Goat anti mouse 2mg/ml, Molecular Probes, A 11003), Connexin-43 1:100 (BD Bioscience C13720-050), Alexa fluor® 488 phalloidin 1:60 (Molecular probes A12379).
Block solution: TBS 0.05 M, pH 7.4, 10% FCS, 1% BSA, 0.5% Triton X-100; antibody solution: TBS 0.05 M, pH 7.4, 1% BSA, 0.5% Triton X-100. To stain whole mount samples incubation time with block solution, first and secondary antibodies was extended to 24 hours at 4°C.
**Force Measurements**

Force of contraction and resting tension of FBMEs were analyzed under electrical stimulation (2 Hz) in thermostatted (37°C) organ baths filled with Tyrode’s solution (mM: NaCl 120, KCl 5.4, MgCl₂ 1.0, CaCl₂ 0.2 to 2.4, NaH₂PO₄ 0.4, NaHCO₃ 22.6, glucose 5.0, Na₂EDTA 0.05, ascorbic acid 0.3) as previously described (1). Inotropic responses to calcium and preload were analyzed as described earlier (2).

**Nucleic acid isolation**

RNeasy® (Qiagen) was used to prepare total RNA. Proteinase K step was incorporated according to manufactures instruction to digest fibrin. DNeasy® Blood & Tissue Kit (Qiagen) was used to prepare genomic DNA according to manufactures instruction. Use of non-failing human heart samples was reviewed and approved by Ethics Committee, University Hamburg.

**Quantification of gene expression**

For reverse transcription and quantitative RT-PCR High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and POWER SYBR® Green PCR Master Mix (Applied Biosystems) were used according to manufactures instructions. Experiments were performed on ABI PRISM 7900HT Sequence detection system (Applied Biosystems). Qualitative PCR amplification of ion channels was performed with OneStep RT-PCR kit (Qiagen) according to manufacturer’s instructions. Primer sequences are listed in Online Table I.

**Measurement of proliferation and apoptosis by ELISA**

Histone preparation was performed with EpiQuik™ Total Histone Extraction Kit (Epigentek OP-0006), phosphorylation of Histone H3 was determined with EpiQuik™ Global Histone H3 Phosphorylation (Ser10) Assay Kit (Epigentek P-7002-48-EP). Caspase-3 activity was determined with CaspSELECT™ Caspase-3 Immunoassay Kit (Biovision K163-100-BV). A 24-well plate of FBMEs was harvested at six different time points after casting (0, 3, 6, 9, 12, 15 days, n=4 each). Protein/histones were prepared according to manufacturer’s instructions, stored at -80 °C and then processed in parallel according to manufacturer’s instructions. HEK293 cell cultures at medium density and 5-azacytidine-treated FBMEs served as positive and negative controls for the histone H3 phosphorylation assay, doxorubicin-treated FBME (10 µM over night) as a positive control for the caspase-3 assay.
Toxicity testing

FBMEs were incubated consecutively with increasing concentrations chromanol 293B (overnight, Sigma Aldrich C2615), erythromycin (overnight, Sigma Aldrich E5389), quinidine (1 hour, Roth 3348.1) and contractility was analysed after each concentration by video optical recording. Cardiotoxic effects of doxorubicin (Sigma Aldrich D1515) were tested by applying the indicated concentrations in parallel series of FBMEs. Contractility was analysed daily by video optical recordings.

Action potential recordings

FBMEs were mounted with two clips in a double-jacketed organ bath and equilibrated in modified Krebs-Henseleit solution containing (in mmol/L): 115 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.2 CaCl₂, 25 NaHCO₃, 10 D-glucose (continuously gassed with carbogen to provide oxygenation and a pH of 7.3–7.4) at 36°C. Chymotrypsin (0.0025%, Sigma-Aldrich C4129) and collagenase CLS type II (0.2 mg/ml; 65 units/ml; Worthington, NY 4176) were added to the bath solution to facilitate impalement of cardiomyocytes within the fibrin matrix. After one hour of exposure to the enzyme mix, spontaneous action potentials were measured with conventional glass capillaries with resistances of 20 to 25 MΩ when filled with 3 M KCl. A pair of platinum wires was used to electrically stimulate FBMEs with square wave pulses of 4 ms in duration, a stimulation frequency of 1 Hz and an intensity slightly above threshold. Transmembrane electrical activity was recorded with an electrometer amplifier (model 773; World Precision Instruments; New Haven, CT) and digitized at 1 kHz via a 16-bit A/D interface. The action potential duration (APD) was measured at 80% repolarisation (APD₈₀). Data acquisition and analysis was performed using an ISO-3 multitasking patch-clamp program (MFK, Niedernhausen, Germany). Further data processing was carried out with SigmaPlot 11.0.
Online Figures and legends

Online Figure I

Histological analysis of FBME development. FBMEs (incubated with aprotinin) were fixed with formaldehyde at indicated time points, embedded in paraffin and sections were stained with H&E, x 10 magnification. A Day 0: cells are present as single, round and amorphous cell suspension in fibrin matrix; B day 3: cells spread out along force lines. C day 6, D day 9: degradation of extracellular matrix, cells spread and align with neighbouring cells. E day 12, F day 15: extended degradation of extracellular matrix, the cellular density is increased; cells align and show orientation along force lines. Scalebar 100 µm.
Online Figure II
DNA- and total RNA content of FBMEs. A DNA content of FBMEs over time (n = 4). B Total RNA content over time (n = 4). * p<0.05 vs. d0 (Students t test). Bars show means +/- SD. C, D Concentration of phosphorylated histone H3 (C) and caspase-3 activity (D) in FBMEs over time of cultivation. Day 0 represents freshly solidified FBME 2 h after casting. Proliferating HEK293 cells and AraC-treated FBME served as positive and negative control for proliferation, respectively. Doxorubicin-treated FBME served as positive control for caspase-3 activity. Bars show mean +/-SEM, n=4. *p<0.05 vs. d0 (Students t test).
Online Figure III
Agarose gel of PCR fragments amplified from FBMEs and non-failing human heart total RNA. Primer sequences and predicted size of PCR fragments are listed in Online Table 1. A potassium channels, B calcium channels, sodium channels.

Online Figure IV
Original mechanogram of FBME (incubated with aprotinin) in organ bath experiment. Inotropic response (twitch tension) to preload (Frank-Starling mechanism) and extracellular calcium concentration were recorded. Maximum twitch tension was approximately 0.9 mN.
Online Figure V
Original contraction peak recordings in the absence and presence of proarrhythmic drugs (please note different time scale).

Quinidine baseline

Quinidine 0.1 µM

Quinidine 1.0 µM

Quinidine 10 µM

Quinidine 100 µM
Chromanol baseline

Chromanol 0.1 µM

Chromanol 1.0 µM

Chromanol 10 µM

Chromanol 100 µM
## Online Table I

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**FBME maturation**

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Supplemental Reference


Legends for video file

Online Movie
FBME contraction (incubated with aprotinin, insulin and tranexamic acid), view from above.