
Contractile three-dimensional bioengineered heart muscle for myocardial regeneration

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Abstract: Tissue engineered heart muscle may be able to provide a treatment modality for early stage congestive heart failure. In this study, we describe a new method to engineer functional 3-dimensional heart muscle utilizing a biodegradable fibrin gel. Primary cardiac myocytes were isolated from hearts of 2- to 3-day-old rats and processed in one of the two ways. For the first method (layering approach), the cells were plated directly on the surface of a fibrin gel-coated on polydimethylsiloxane (PDMS) surfaces. The cells were cultured in growth media and the contractile performance evaluated after formation of 3-dimensional tissue constructs. For the second method (embedding approach), the cells were suspended with thrombin and plated on 35 mm tissue culture surfaces coated with PDMS. Fibrinogen was then added to the surface. Within 7 days after initial cell plating, a 3-dimensional tissue construct of cells derived from primary heart tissue (termed bioengineered heart muscle, BEHM) resulted for both approaches.

Histological evaluation showed the presence of uniformly distributed cardiac cells throughout the BEHM, both in longitudinal and cross sections. The stimulated active force of BEHMs formed using the layering approach was $835.5 \pm 57.2 \mu\text{N}$ ($N = 6$) and $145.3 \pm 44.9 \mu\text{N}$ ($N = 6$) using the embedding approach. The stimulated active force was dependent on the initial plating density. It was possible to maintain the contractile function of BEHM in culture for up to 2 months with daily medium changes. The BEHMs exhibited inotropy in response to external calcium and isoproterenol and could be electrically paced at frequencies of 1–7 Hz. We describe a novel method to engineer contractile 3-dimensional cardiac tissue construct with a fourfold increase specific force compared to our previous model. © 2006 Wiley Periodicals, Inc. *J Biomed Mater Res* 80A: 719–731, 2007

Key words: cardiac myocytes; fibrinogen/fibrin; tissue engineering; contractile function; force

INTRODUCTION

Tissue engineering is based on the assumption that patient-derived cells can be expanded in culture without loss of differentiated phenotype to form functional 3-dimensional tissue constructs.^{1,2} Patient-derived primary cells can be engineered to form functional 3-dimensional cardiac muscle in the presence of the appropriate electro-mechanical and chemical interventions. Such tissue engineering methodology could be utilized in translating 2-dimensional monolayer cell cultures to more physiological 3-dimensional tissue engineered constructs. Potential applications for 3-dimensional tissue engineered constructs would be in high throughput drug screening and clinically as patches to augment failing myocardial function.³

There have been several strategies utilized to engineer cardiac tissue *in vitro*. Synthetic scaffolds,⁴ biodegradable gels,^{5–7} and self organization strategies⁸ have all been evaluated. Biodegradable gels were the first model utilized to engineer functional 3-dimensional heart muscle *in vitro*.⁹ Eschenhagen developed a 3-dimensional model of tissue engineered heart muscle by culturing chick cardiac myocytes in a collagen matrix.⁹ The initial model was refined to generate engineered heart tissue (EHT) by casting a mixture of neonatal cardiac myocytes and collagen into plastic molds.¹⁰ The EHTs were capable of generating a stimulated active force of up to $500 \mu\text{N}$ ¹⁰ and exhibited several physiologically (contractile, histological, and biochemical) metrics of heart function.¹⁰ Carrier et al. were the first to demonstrate the feasibility of utilizing polymeric scaffolds for cardiac tissue engineering application.⁴ They utilized fibrous meshes made from polyglycolic acid as a scaffolding material for culturing neonatal cardiac myocytes.⁴ Okano developed sheets of cardiac cells in the absence of any synthetic scaffolding material to promote the formation

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of functional 3-dimensional cardiac muscle *in vitro*.^{11,12} Temperature sensitive surfaces have been used to manufacture 2-dimensional sheets of cardiac myocytes.^{13–16} These sheets have been stacked together to form 3-dimensional cardiac muscle.¹⁵

Our previous work has focused on promoting the self organization of primary cardiac cells to form contractile 3-dimensional heart muscle (cardioids) *in vitro*.⁸ Cardioids were formed from the spontaneous delamination of a confluent monolayer of neonatal cardiac myocytes.⁸ Cardioid formation depends on the self-organization of individual cardiac cells into a 3-dimensional tissue construct.⁸ This process eliminated the need for synthetic scaffolding material in the contractile region of the tissue construct. Our initial work demonstrated the feasibility of utilizing self-organization strategies to engineer functional heart muscle *in vitro*. Recently, we have evaluated the use of a biodegradable fibrin gel to promote the formation of 3-dimensional tissue constructs.¹⁷ The major advantage of utilizing fibrin gel is the easily controllable degradable kinetics and the rate of fibrin degradation can easily be matched with the formation of new tissue. We have successfully used this strategy to engineer contractile skeletal muscle tissue *in vitro*.¹⁷

The current state of the art in cardiac tissue engineering allows the formation of 3-dimensional patches of heart muscle utilizing hydrogels, polymeric scaffolds, and self organization strategies. It would be interesting to compare these three different strategies. Self organization strategies have the advantage of stimulating cardiac cells to generate their own extracellular matrix components, whereas hydrogels replicate the cardiac ECM with commercially purified sources. However, the major limitation of both these strategies is that the resulting constructs often lack the mechanical strength required to sustain physiological conditions. Polymeric scaffolds satisfy the requirement of providing a closely match and easily controllable mechanical environment for cardiac tissue constructs. Scaffolds also offer the advantage of customized patches and provide an opportunity to engineer bioartificial ventricles, which would be more challenging with hydrogels and self organization methods.

We believe that each strategy offers several advantages towards engineering functional 3-dimensional heart muscle *in vitro*. Our laboratory is focused on engineering functional heart muscle utilizing three different strategies (self organization, hydrogels and scaffolding) and understanding the unique features of each model.

In this study, we were interested in evaluating the feasibility of utilizing a rapidly degrading fibrin gel to engineer functional cardiac muscle *in vitro*. The objective of this study was to evaluate the feasibility of utilizing fibrin gel as a support matrix to promote

the formation of 3-dimensional heart muscle. We describe methodology for the formation of bioengineered heart muscle and metrics of myocardial performance of the resulting tissue engineered construct.

METHODS

NIH guidelines for the care and use of laboratory animals (NIH Publication no. 85-23 Rev. 1985) have been observed. All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Isolation of neonatal cardiac myocytes

Cardiac myocytes were isolated from 2- to 3-day-old F344 rat hearts using an established method.¹⁸ Hearts were cut into fine pieces and suspended in a dissociation solution (DS) that consisted of 0.32 mg/mL collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) and 0.6 mg/mL pancreatin dissolved in a buffer consisting of 116 mM NaCl, 20 mM HEPES, 1 mM Na₂HPO₄, 5.5 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO₄. Digestion was carried out in an orbital shaker for 5 min at 37°C, after which the supernatant was replaced with fresh DS and the digestion process was continued for an additional 30 min. At the end of the digestion process, the supernatant was collected in 5 mL of horse serum (Invitrogen Corporation, Auckland, New Zealand), centrifuged at 1500 rpm for 5 min and the cell pellet was resuspended in 5 mL horse serum. Fresh DS was added to the original, undigested tissue and the digestion process was repeated an additional 2–3 times. Cells from all the digests were pooled, centrifuged, and then suspended in culture medium (CM) consisting of 320 mL M199, 100 mL F12k, 50 mL fetal bovine serum, 25 mL horse serum, 5 mL antibiotic-antimycotic (Invitrogen Corporation), hydrocortisone 40 ng/mL, and insulin 100 ng/mL.

Preparation of the plates

The detailed method for preparing the culture surface for engineering skeletal muscle has been described in detail.^{17,19} This procedure was modified to engineer cardiac muscle.⁸ Briefly, 35-mm culture plates were coated with 1.5 mL of a polydimethylsiloxane (PDMS) elastomer (Dow Chemical Corporation, Midland, MI). Anchor points were 6-mm long segments of size 0 braided silk sutures (Ethicon, Cornelia, GA) pinned 12 mm apart in the center of the culture surface.

Formation of fibrin gel and cell plating

For layering approach, 0.5 mL of CM containing 10 U/mL thrombin were plated on the surface of 35-mm cell culture plates coated with SYLGARD (PDMS, type 184 silicone elastomer) [Fig. 1(A)]. After this, 200 μ L of 20 mg/mL fibrinogen was added to the 35-mm plate. The solution was mixed to promote the formation of a fibrin gel within 10–15 min. Primary cardiac cells were diluted in CM at varying densities (0.5, 1, 2, and 4 millions/plate) and plated in 1 mL of CM for each plate after complete gel formation.

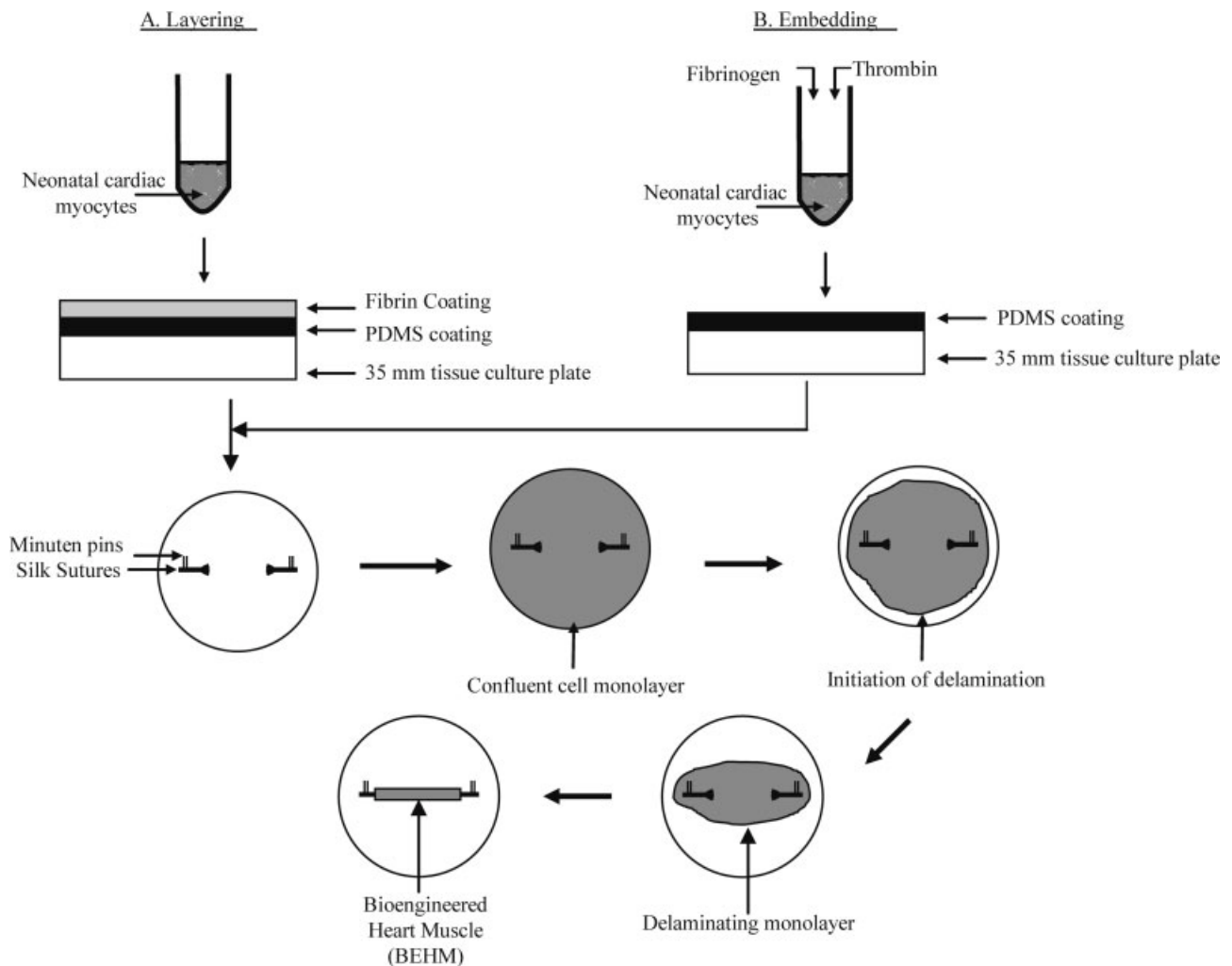


Figure 1. Methodology for the formation of bioengineered heart muscle (BEHM). A: Layering approach. Tissue culture plates are coated with PDMS followed by thrombin and then fibrinogen. Neonatal cardiac myocytes are then plated on the surface of the fibrin gel. B: Embedding approach. Neonatal cardiac myocytes are mixed with fibrinogen and then thrombin. The cell suspension is then plated on the PDMS surface. In both cases, the spontaneous contractility of the neonatal cardiac myocytes results in delamination of the cell monolayer. Delamination initiates at the periphery of the culture and progresses towards the center of the culture surface. The cell monolayer attaches to anchor points that have previously been placed at the center of the culture surface.

For the embedding approach, cells were suspended in 0.5 mL medium containing 10 U/mL thrombin and plated on the surface of 35-mm cell culture plate coated with SYLGARD [Fig. 1(B)]. Following cell plating, 200 μ L of 20 mg/mL fibrinogen was added to the plate. The solution on the surface of each plate was gently mixed by lateral agitation, promoting the formation of a fibrin gel within 10–15 min. After complete gel formation, 1 mL of CM was added to each plate.

The cells were cultured in an incubator at 37°C and 5% CO₂ with medium changes every second day.

Evaluation of contractility

The method for evaluating the contractility of engineered skeletal and cardiac muscle has been described in

detail.⁸ This method was modified slightly. Briefly, the bioengineered heart muscle (BEHM) was placed in CM at 37°C between parallel platinum electrodes. One end was fixed to the plate and the other end was attached to a custom-built optical force transducer. Stimulated active force (response to a single electrical impulse) measurements were recorded at 15 V, with a frequency of 1 Hz and a 10 ms pulse width. The length of each BEHM was adjusted to obtain maximum stimulated active force using a multi-axis micromanipulator. This optimal length was designated as L_0 and was recorded. The cross-sectional area of the BEHMs was calculated from the construct diameter that was determined by a calibrated eyepiece reticle with a resolution of 5 μ m. The construct was assumed to be cylindrical for this calculation. The specific force (kN/m²) of each BEHM was determined by normalizing the stimulated twitch force to the total cross-sectional area.

Initial stimulation parameters were selected based on our previous experience with engineered cardiac muscle constructs. The stimulation parameters had been optimized for this system in pilot experiments. The BEHM was electrically paced at frequencies between 1 and 7 Hz, with all other stimulation parameters remaining constant.

The effect of the addition of calcium on contractility was investigated in six constructs. For this part of the testing, the constructs had been in culture for 7 days after initial cell plating. The constructs were only used to evaluate the calcium sensitivity with no additional testing being performed. First, CM in the bath was replaced with calcium-free Tyrode's solution. Known volumes of 100 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were then added to the bath to bind residual stores of calcium in the constructs. At each incremental addition of EGTA, twitch stimulation was applied and the stimulated active force of contraction was measured. Removal of calcium was considered to be complete when the stimulated active force of contraction was not detectable and no further EGTA was added. At this point, the calcium-free DMEM in the bath was aspirated and the construct was washed three times with fresh calcium-free DMEM. Known volumes of 100 mM calcium were then added and stimulated active forces were re-evaluated with each addition. This process was repeated until there was no further increase in the stimulated active force with the addition of calcium. For each construct, the stimulated active force was normalized to the maximum stimulated active force generated for the particular construct. An average normalized value was obtained for the six constructs and plotted against the calcium concentration.

The response to isoproterenol was studied in six constructs by adding known concentrations of isoproterenol to the Tyrode's solution in the presence of 100 μ M calcium (ED_{50} , effective dose for 50% of the maximum twitch force from previous calcium study), with the constructs still attached to the force transducer. We evaluated the stimulated active force after each incremental addition of isoproterenol. Once again, the stimulated active force was normalized to the maximum stimulated active force generated for the particular construct and an average normalized value plotted against the isoproterenol concentration.

Histology

Following force testing, constructs were fixed in 4% paraformaldehyde for 4 h and stored in 70% ethanol. The constructs were prepared in an automated tissue processor (Shandon Hypercenter XP, Thermo Electron, Waltham, MA) and then paraffin embedded. Seven micron sections were cut and placed on Probeon Plus slides (Fisher Scientific Company, Pittsburg, PA). Hematoxylin and eosin (H&E) staining was used for morphologic analysis of the constructs.

Immunohistochemistry

Samples (whole hearts and BEHMs) were washed with 1 \times DPBS 3 min and placed into a 10% formaldehyde

solution for 1 h. Samples were then washed with 1 \times DPBS for 3 h and placed in 30% sucrose overnight. Samples were dried and placed in a plastic peel-away container filled with OCT compound and frozen on dry ice, wrapped in parafilm, and stored at -80°C until use. The samples were sectioned at 10 μm using a cryostat set at -20°C and kept frozen (-80°C) until ready for staining. For immunohistochemistry, the sections were blocked with a blocking solution containing 2% BSA, 2% normal goat serum, 0.1% triton X-100, and 0.05% tween 20 for 1 h at room temperature and then stained with the primary antibody for 1 h. The dilution ratios were as follows: 1:250 for polyclonal anticollagen type I (Chemicon International, Temecula, CA, Cat no. AB755P), 1:500 for monoclonal anti- α -sacromeric actin (Sigma, St. Louis MO, Cat no. A2172), 1:50 for connexin43 (Chemicon International, Temecula, CA, Cat no. AB1727) and 1:50 for monoclonal anti-*N*-cadherin/A-CAM clone GC-4 (Sigma, St. Louis, MO, Cat no. C3865). The sections were then washed with 1 \times DPBS three times for 5 min and then incubated with secondary antibody for 30 min in the dark. For the secondary antibodies, we used anti-mouse secondary IgG FITC conjugate (Sigma, St. Louis, MO, Cat no. F5262) at a 1:150 dilution in 1 \times DPBS for anti-*N*-cadherin and anti- α -sacromeric actin and anti-rabbit secondary Cy3 conjugated (Chemicon International, Temecular, CA, Cat no. AP132C) at a dilution of 1:250 in DPBS for anticollagen type I and anticonnexin43. The sections were then washed with 1 \times DPBS three times for 5 min and air dried. A few drops of prolong gold antifade reagent with DAPI (Molecular Probes, Eugene, OR) were then added to the sections, which are covered with a coverslip prior to viewing.

Statistical analysis

We used one way ANOVAs combined with the Tukey's test for all pair-wise comparisons. Minitab V13.31 (State College, PA) was used for statistical analysis.

RESULTS

We engineered 3-dimensional heart muscle utilizing both the layering and the embedding strategies. There was a 100% success rate for construct formation with an excess of 50 constructs formed by each method.

There were several notable methodology differences during BEHMs formation between the two different approaches. We observed differences in the degree of cell spreading utilizing the embedding approach *versus* the layering approach. When the cardiac cells were embedded in the fibrin gel, there was a significantly lower degree of cell spreading as compared to cardiac cells plated on the surface of the fibrin gel.

We also observed differences in the degree of spontaneous contractility of the cell monolayer prior to the formation of a 3-dimensional construct. Within

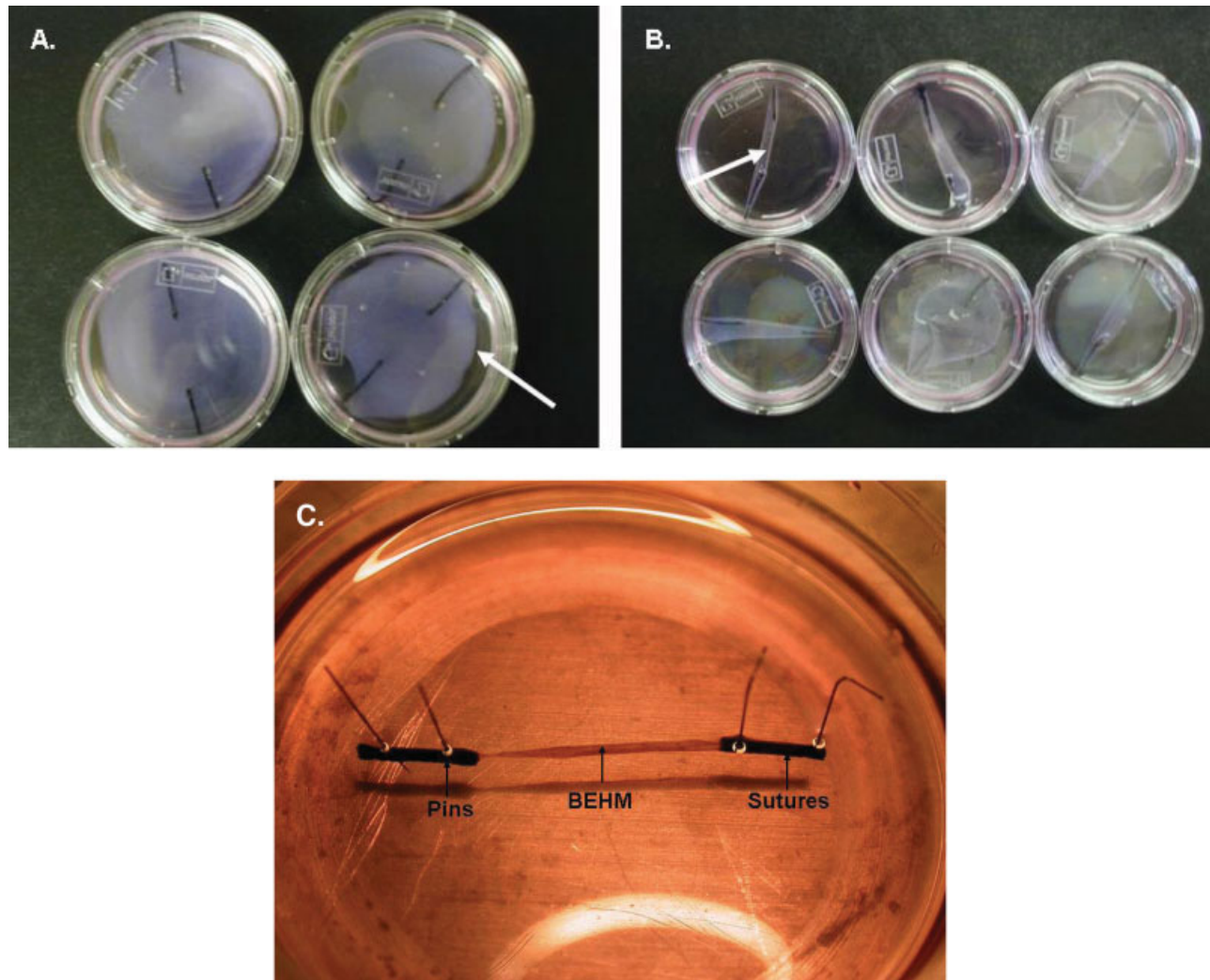


Figure 2. Various stages of formation of bioengineered heart muscle. Within 3 days after plating, the neonatal cardiac myocytes initiate spontaneous contractility. The contractility of the cell monolayer initiates delamination (arrows) of the fibrin gel from the underlying culture surface. Delamination is initiated at the (A) periphery of the culture surface and progresses towards the (B) center of the culture surface. (C) After complete formation, the bioengineered heart muscle (BEHM) is suspended between anchor points that have been pinned to the culture surface with 0.1 mm pins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

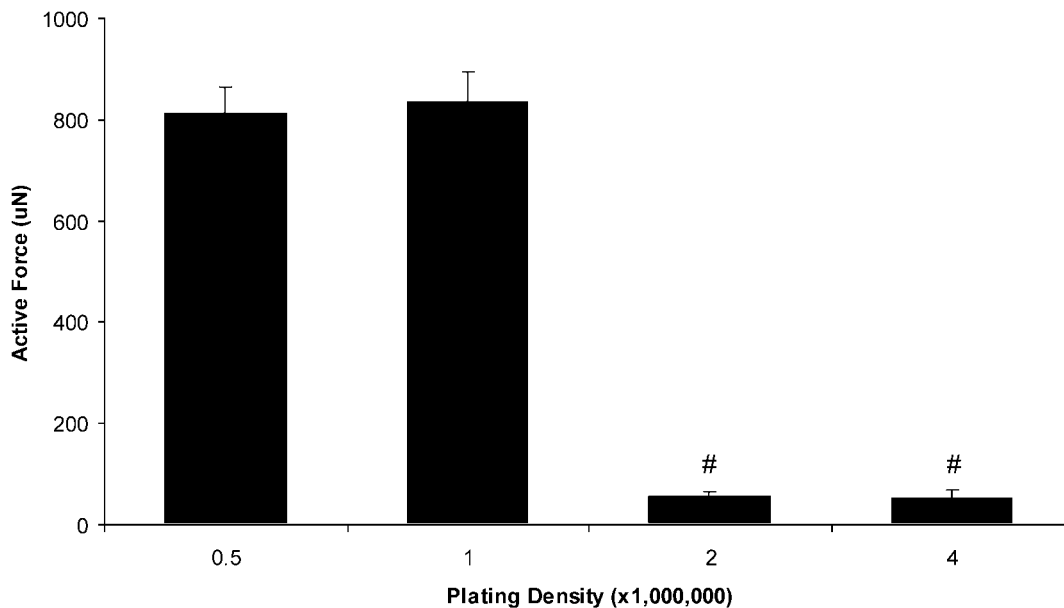
48 h after initial cell plating, we were able to observe spontaneous contractions of the cell monolayer in the layering approach. The rate of spontaneous contractions of the cell monolayer was in the order of 1–2 Hz and was very regular and consistent for the duration of cell culture. In contrast, we only observed the spontaneous contractions of clusters of cardiac cells trapped in fibrin gel for the embedding approach. These clusters of cardiac cells were uniformly distributed throughout the fibrin gel.

Although we observed differences in monolayer contraction and degree of cell spreading, there were no notable differences during compaction of the fibrin gel between the two approaches. Within 3–5 days after initial cell plating, we observed compaction of the cell–gel construct for both approaches.

Compaction of fibrin gels was initiated at the periphery of the culture surface due to the traction force from cells and the degradation of the fibrin gel [Fig. 2(A)]. This process continued to progress towards the center of the culture surface and was aided by the spontaneous contractions of the cardiac myocytes [Fig. 2(B)]. At the center of the plate, the cell–gel constructs attached to the anchor points that were previously pinned into the center of the culture plate [Fig. 2(C)]. The basal tension was maintained between both sutures resulting in the formation of a 3-dimensional heart muscle construct, which we have termed bioengineered heart muscle (BEHM) [Fig. 2(C)].

We evaluated the effect of cell density on the contractile performance of BEHM formed by the embed-

A. Layering Approach



B. Embedding Approach

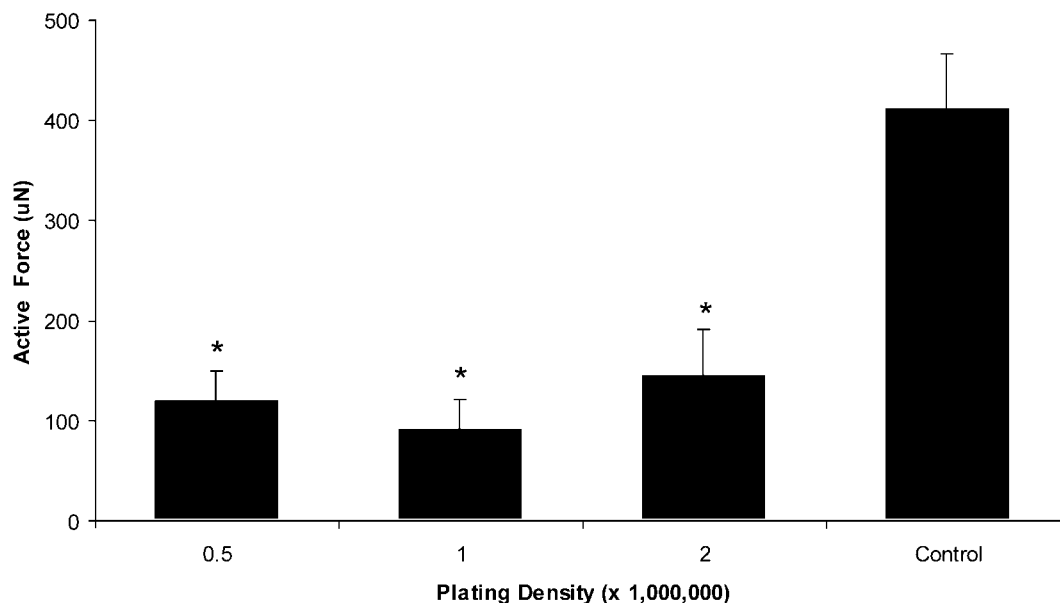


Figure 3. Effect of cell density on the functional performance of BEHM. A: Embedding approach. Varying cell numbers were suspended in a fibrin gel and utilized to form BEHM. We utilized cell numbers of 0.5 , 1.0 , and 2.0×10^6 cells per plate. As controls, we utilized the layering approach for BEHM formation with a plating density of 0.5×10^6 cells per plate. * denotes a statistical significant difference from controls with $p < 0.05$. There was no significant difference between the three groups. B: Layering approach. We utilized plating density of 0.5 , 1.0 , 2.0 , and 4.0×10^6 per plate. The stimulated active force was evaluated within 48 h of BEHM formation. For each group $N = 6$ BEHM. For every BEHM utilized utilizing both methods, we obtained 4 active contractions and used the average value for each BEHM. # denotes a statistical difference from the 0.5 and 1.0 group for the layering approach with $p < 0.05$. There was no significant difference between the 0.5 and 1.0 group as well as the 2.0 and 4.0 group for the layering approach.

ding approach as well as the layering approach (Fig. 3). The stimulated active force for constructs formed by the embedding approach was found to be in the range of 102 – 220 μN [Fig. 3(A)], whereas the

stimulated active force for constructs formed by the layering approach was found to be in the range of 36 – 923 μN [Fig. 3(B)]. We found that there was no significant change in the contractile performance of

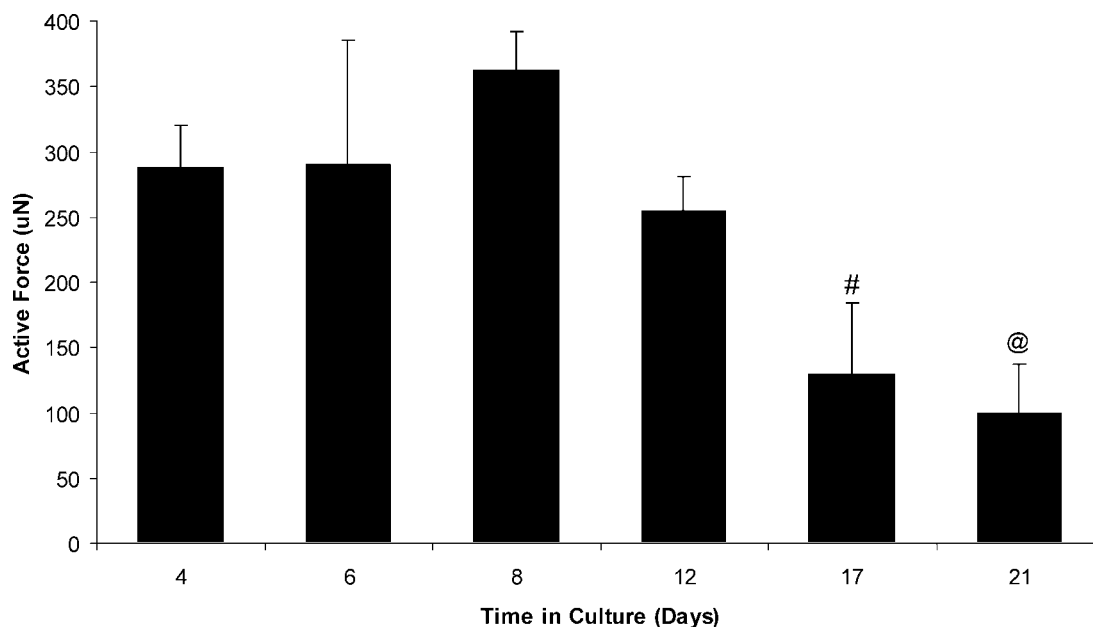


Figure 4. Changes in contractile performance of BEHM in culture. We evaluated the stimulated active force of BEHM that was formed using the layering approach with a plating density of 1×10^6 cells/constructs at 4, 6, 8, 12, 17, and 21 days after complete formation. For each group, $N = 6$ BEHMs and for each BEHMs we use an average of 4 twitches. There was no statistical difference between the stimulated active force at 4, 6, 8, and 12 days. # denotes a significant difference with 4, 6, 8, and 12 day BEHM. @ denotes no significant difference with 17 day BEHM. For all cases, $p < 0.05$).

BEHMs formed by the embedding approach utilizing a cell density of 0.5×10^6 – 2.0×10^6 cells per plate [Fig. 3(A)]. In the case of BEHMs formed using the layering approach, lower plating density resulted in a higher contractile performance [Fig. 3(B)]. We obtained the maximum stimulated active force of 812.3 ± 54.2 μN utilizing a plating density of 0.5×10^6 – 1.0×10^6 cells per plate for the layering approach. There was a significant decrease in the stimulated active force of the BEHM when the plating density was increased beyond 1.0×10^6 cells per plate [Fig. 3(B)]. We also obtained a significant decrease in the stimulated active force of BEHM formed using a plating density lower than 0.5×10^6 cells per plate for the layering approach (data not shown).

Based on our findings that BEHMs formed by the layering approach generated significantly higher forces than BEHMs formed by the embedding approach, we decided to proceed with further investigation using the former (layering) approach.

We evaluated the changes in contractile performance of BEHM over time in culture (Fig. 4). Utilizing a plating density of 1×10^6 cells/construct, we found that the BEHMs were able to maintain contractile performance over a period of 12 days, after which time we observed a significant drop in the stimulated active force. In subsequent experiments, we were able to maintain the ability to generate stimulated active force of the BEHM with daily medium changes for a period of up to 2 months (data

not shown). During long-term culture of BEHM, there was a significant decrease in the diameter of the construct over time. At the time of formation (4–5 days), the diameter of the BEHM was larger than 500 μM . After one week in culture, the diameter decreased to 200 – 300 μM , and to an average value of 150 – 250 μM after 2 weeks in culture. We calculated the specific force of the constructs by normalizing the stimulated active force to the total cross-sectional area. At the time of formation, the specific force of the constructs was found to be 2 – 4 kN/m^2 . After 2 weeks in culture, though the absolute force did not increase, the specific force increased to 12 – 15 kN/m^2 due to the decrease in cross sectional area of the BEHM, and the specific force was maintained for the duration of the culture.

We also evaluated the changes in passive force of the BEHMs over time and found that the average passive force was 100 – 200 μN and did not change significantly over time. The passive force was due to the baseline activity of the cells while fibrin degradation did not significantly influence the passive force. Therefore, the passive force did not change significantly over time, during which period there was degradation of the fibrin gels.

Hematoxylin and eosin staining was utilized to study the rate of fibrin degradation as well as the cellular distribution within the 3-dimensional BEHM. At the time of formation, greater than 50% of the construct volume was occupied by the fibrin gel [Fig. 5(A)]. During culture, the fibrin gel was

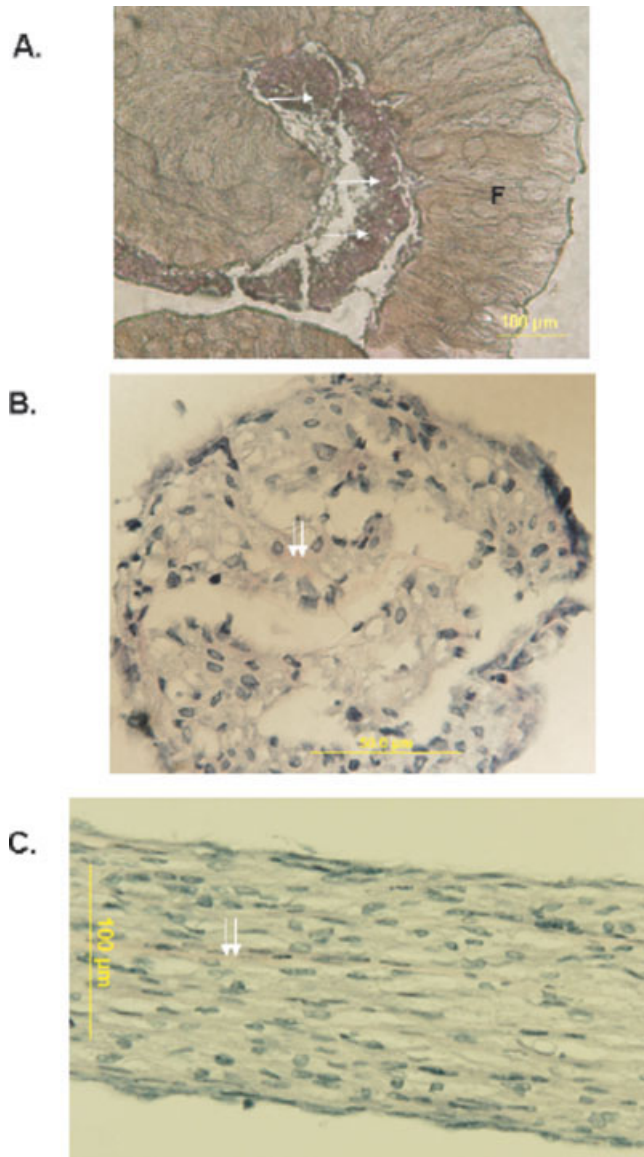


Figure 5. Histological characterization of BEHM. A: The constructs (made by the layering approach) were stained with hematoxylin and eosin 2 days after complete formation. At 2 days, there was a significant amount of fibrin (F) in the construct with scattered cardiac cells (single arrow). For (B) and (C), the constructs were stained with hematoxylin and eosin 1 week after complete formation. B: Cross-section shows the presence of uniformly distributed cells throughout the 3-dimensional tissue construct. C: Longitudinal section shows a very high degree of cellular alignment. In both cases, non degraded fibrin is visible (double arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degraded by the cardiac cells, which resulted in a smaller volume of the BEHM being occupied by the cells. A significant percentage of the fibrin was degraded within 7 days and there was virtually no fibrin visible by day 14 [Fig. 5(B)]. The total volume of engineered heart muscle constructs was reduced from initial 700 mm³ (0.7 mL fibrin gel) to 0.4 mm³

(200 μm diameter of 12-mm constructs) after 2-week culture.

The cardiac cells were uniformly distributed throughout the BEHM. This was evident in both the cross-section [Fig. 5(B)] as well as the longitudinal section [Fig. 5(C)] of the BEHM. In the cross-section, we observed a relatively uniform distribution of cells throughout the BEHM. In addition, a very small amount of fibrin was also evident. This was also the case for the longitudinal section. The cardiac cells were aligned along the length of the BEHM. The degree of alignment was very regular throughout the length of the construct.

We evaluated the distribution of collagen type I, F-actin, connexin43, and *N*-cadherin in the cardioids (Fig. 6). The collagen appeared to be present in an early unpolymerized state with very little evidence of well-formed fibers [Fig. 6(E)]. The F-actin seemed to be present in large amounts with longitudinally aligned fibers visible [Fig. 6(F)]. Staining for connexin43 [Fig. 6(G)] and *N*-cadherin [Fig. 6(H)] showed the formation of electrical and mechanical coupling points between adjacent cardiac myocytes.

We evaluated the pacing characteristics of the BEHMs (Fig. 7) at frequencies of 1, 3, 5, and 7 Hz. At the lower pacing frequencies, the BEHMs were able to return to baseline force between active contractions. In addition, there were no signs of fatigue (as evaluated by the size of successive contractions) and we observed a direct correlation between the number of electrical impulses and the number of stimulated active contraction. At the higher frequencies (5 and 7 Hz), there was also a linear correlation between electrical impulses and number of contractions with no signs of fatigue. However, the constructs were not able to return to baseline conditions between stimulated active contractions at the higher frequencies.

We evaluated the responsiveness of the BEHM to calcium (Fig. 8). In the absence of calcium or at very low calcium concentrations, the BEHMs were not able to generate measurable stimulated active force. However, as the bath calcium concentration was increased, we observed a near linear increase in the stimulated active force. The stimulated active force increased to 50% of maximal value at a calcium concentration of 100 μM (ED₅₀) and 90% within the range of 800–1200 μM. The pCa_{1/2}, defined as the negative log of the calcium concentration required to generate half the maximal force, of our BEHMs was found to be in the range of 4.0–4.2.

We also evaluated the effect of isoproterenol on the contractility of BEHM (Fig. 9). We obtained a dose-responsive increase in the stimulated active force with increasing concentrations of isoproterenol in the range of 1 nM to 50 nM. We obtained 50% of the maximal stimulated active force at an isoproterenol concentration of 1–5 nM and 90% at 50 nM.

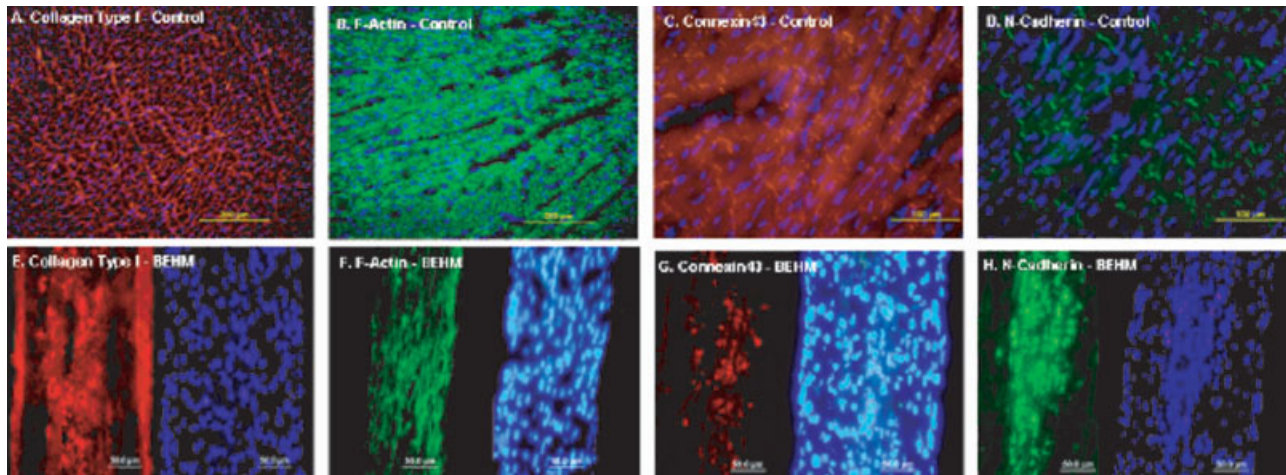


Figure 6. Staining for Collagen type I, F-actin, connexin43, and N-Cadherin. (A–D) Control hearts and (E–H) BEHMs. All images were counterstained with DAPI to visualize the nucleus. For the control hearts, the DAPI image was overlaid with the image for the specific protein. For the BEHMs, the DAPI image is shown separately as the proportion of the specific protein is considerably lower than the nuclear material. The BEHMs used for staining were formed via the layering approach and maintained in culture for 7 days after complete formation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In this study, we evaluated two methods (layering *versus* embedding) to promote the formation of functional 3-dimensional BEHM. Initially, we utilized

fibrin gel as a support matrix to culture skeletal muscle cells.¹⁷ We compared both the layering and embedding approach and found that the layering approach was more suitable to promote myotube formation.¹⁷ Though we found the layering approach

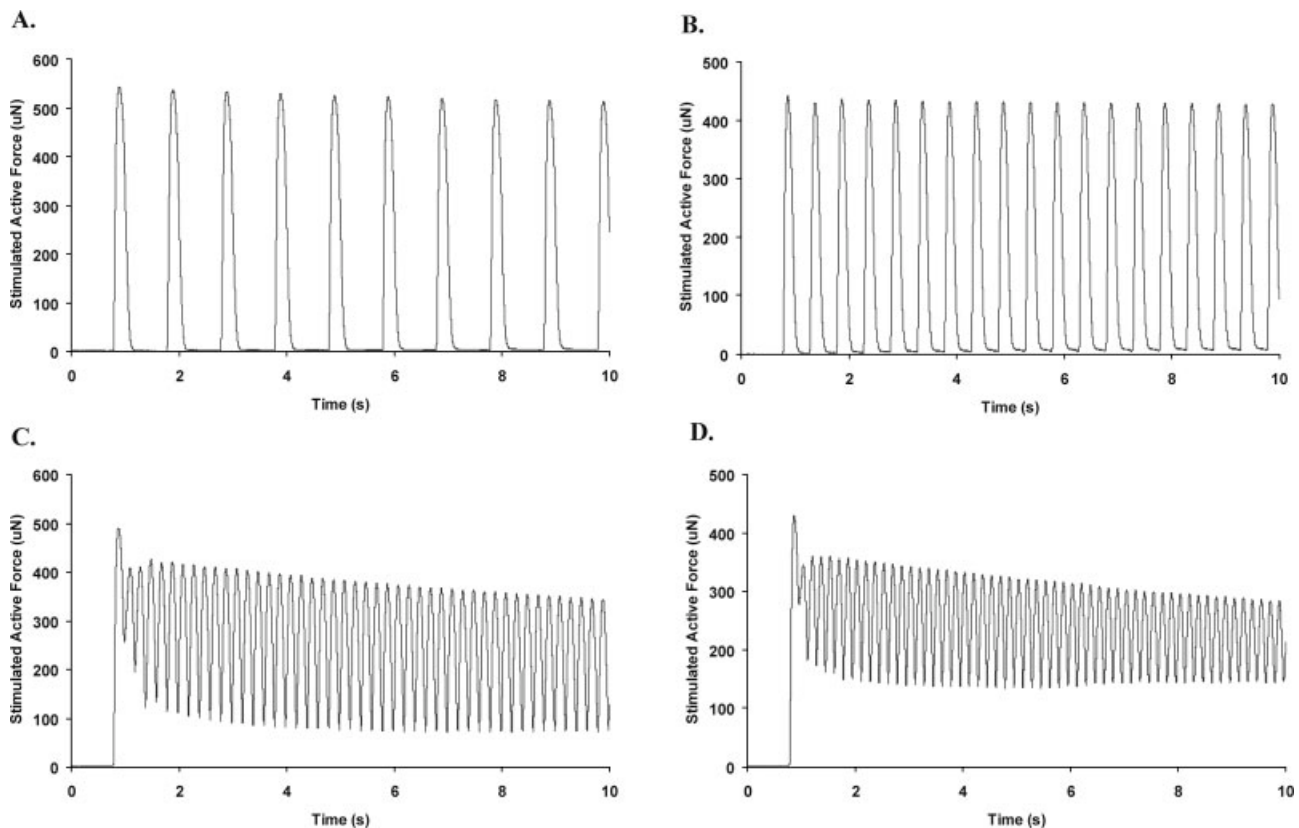


Figure 7. Pacing characteristics of BEHM. The constructs were electrically paced at (A) 1 Hz, (B) 3 Hz, (C) 5 Hz, and (D) 7 Hz. We utilized a voltage of 10 V and a pulse width of 2 ms.

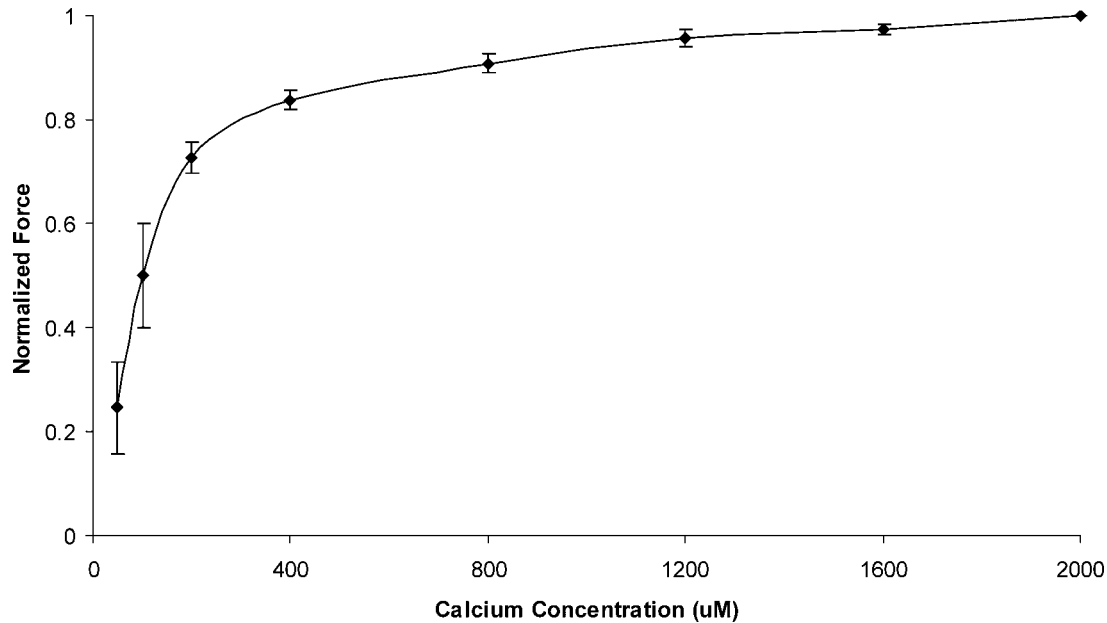


Figure 8. Calcium sensitivity of BEHM. The constructs were placed in a calcium-free DMEM. Calcium was gradually added back to the bath by introducing incremental volumes of 100 mM calcium. The bath concentration of calcium was varied from 50 to 2000 μM . The stimulated active force of contraction was evaluated at each calcium concentration. The stimulated active force at each calcium concentration was normalized to the maximum stimulated active force obtained for the construct. This process was repeated for a total of 6 constructs. The values plotted are the average and standard deviation for the 6 constructs tested. * denotes significant difference in the average stimulated active force at the specified calcium concentration than the average stimulated active force at the previous calcium concentration at $p < 0.05$).

to perform well with skeletal myoblasts, the embedding approach seems to have been a preferred model for other groups in the field.^{4,9} Therefore, we were interested in comparing the two methods to support the formation functional cardiac muscle *in vitro*.

In the case of the embedding approach, primary cardiac cells were suspended in thrombin solution that had been plated on a tissue culture surface were mixed with a fibrinogen solution. For the layering strategy, the fibrin gel was layered on the culture surface and then the cardiac cells were plated on the surface of the fibrin gel. When the cardiac cells are plated on the surface of the fibrin gel, we observed very regular spontaneous contractions of the entire cell monolayer, initiating within 48 h after cell plating. The spontaneous contractions of the constructs resembled the synchronous contractions of the normal myocardium. The spontaneous contractions of the cohesive cell monolayer in synchrony do provide some indirect support for the formation of intercellular connections between adjacent cardiac myocytes as well as connections between the cardiac cells and the fibrin gel. This was not the case for BEHMs formed by the embedding approach, in which case we observed isolated patches of contractile tissue. We speculate that this was likely due to the inability of the cardiac cells to

spread and form intercellular connections with neighboring cells.

We found an interesting response of the BEHMs with plating densities. When utilizing the embedding approach, the plating density (0.5×10^6 – 2.0×10^6 cells/construct) did not affect the stimulated active force production of the constructs. We speculate this was due to the limited cell spreading ability within the fibrin gel. However, when we utilized the layering approach, we found that lower plating densities (0.5×10^6 – 1.0×10^6 cells/construct) resulting in constructs that generated significantly higher forces than constructs formed with higher plating densities (2.0×10^6 – 4.0×10^6 cells/construct). We believe that our static *in vitro* cell culture system is not able to support the viability of larger cell numbers, therefore resulting in lower functional performance. As efficient perfusion systems are developed, there may be a reversal of this trend.

There have been several models described in the literature and the contractile properties of EHT have been described in detail.^{9,20} EHTs are generated by casting a suspension of cardiomyocytes in a collagen gel.²⁰ EHTs have been shown to generate a stimulated active force of 350 μN with a specific force of 0.4 kN/m^2 under basal conditions.²⁰ In comparison, BEHMs have been shown to gener-

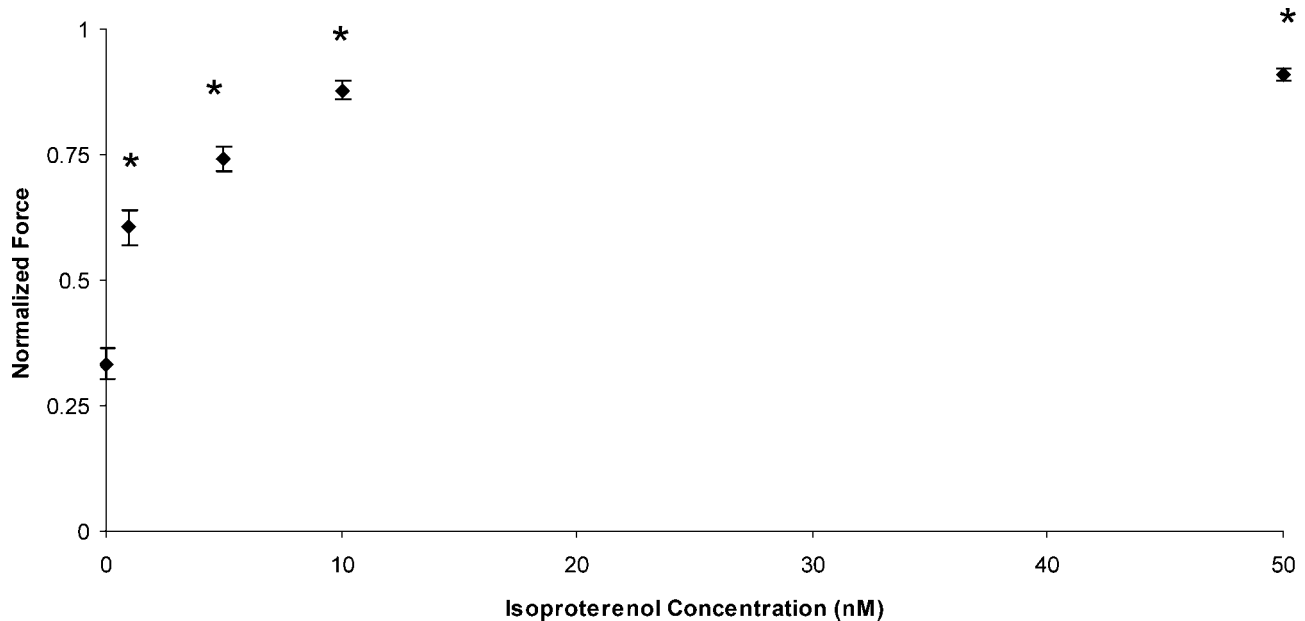


Figure 9. Effect of isoproterenol on BEHM. The constructs were placed in calcium free DMEM. The bath calcium concentration was adjusted to 100 μM by the addition of external calcium. The concentration of isoproterenol was increased from 1 to 50 nM and the stimulated active force evaluated at every incremental addition. The stimulated active force was normalized to the stimulated active force obtained for the construct. This process was repeated for a total of 6 constructs. The values plotted are the average and standard deviation for the 6 constructs tested. * denotes a significant difference from control (no isoproterenol) with $p < 0.05$. # denotes no change in stimulated active force production from previous isoproterenol concentration).

ate stimulated active force in the order of 800 μN and a specific force of 15 kN/m^2 . BEHMs are capable of generating considerably higher active forces under basal conditions than EHTs. We speculate that this is due to a high degree of cellular alignment and compaction without excess extracellular matrixes in the BEHMs after fibrin degradation. The passive tension maintained between the suture materials promotes the alignment of the cardiac cells during BEHM formation, as evident in the histology. In contrast, the EHTs are formed by casting a mixture of cardiac cells with collagen gel. The final EHTs still have a lot of collagen surrounding, which results in large diameter and low specific force.

The specific force of native very thin cardiac muscle preparations is between 20 and 45 kN/m^2 and is dependent on frequency.²¹ For example, the contractile performance of adult rat papillary muscle has been shown to be in the order of 8130 μN for the stimulated active force and a specific force of 44.4 kN/m^2 .²² In comparison to these published values, our BEHMs generate considerable lower specific forces. The maximum specific force generated by BEHMs is in the order of 15 kN/m^2 at the time of complete fibrin degradation. Understanding and optimizing BEHM formation will help bridge the gap between the contract-

ile performance of BEHM and normal myocardial tissue.

Self-organized heart muscle (cardioids) have been shown to generate a stimulated active force of 75–125 μN at the time of formation for a specific force of 2.4–4.0 kN/m^2 .⁸ Clearly, BEHMs generate more stimulated active force than cardioids. We speculate that the fibrin gel simulates a temporary extracellular matrix allowing functional organization of the cardiac cells. Cardioids are self organized from isolate primary cardiac myocytes and fibroblasts.⁸ Although the cardiac fibroblast generate extracellular matrix to support 3-dimensional tissue formation, the degree of cellular organization may not be optimal. This in turn may result in lower active force production.

Histological data provided evidence for some degree of functional coupling between adjacent cardiac myocytes. There was positive staining for connexin43 and N-cadherin that shows some degree of cellular coupling. There was also positive staining for collagen type I and F-actin, showing some degree of tissue level organization of the isolated cardiac cells. Comparison of the BEHM images with those of control hearts provides a clear picture of where we are with the current model and where we need to go. There is still a significant amount of work that needs to be done to bridge the gap

between tissue engineered constructs and normal heart tissue. This is somewhat expected as our tissue engineering models are still in a premature state of development.

In addition to the stimulated active force, we evaluated the pacing characteristics of the BEHM as well as the responsiveness to ionic calcium and isoproterenol. The responsiveness of the BEHM to calcium shows the presence of a functional calcium handling cascade and provides further support for the formation of functional viable heart muscle. The $pCa_{1/2}$ of the BEHMs was found to be in the range of 4.0–4.2. The $pCa_{1/2}$ of mammalian cardiac tissue has been reported in the range 5.9 to 7.0.²³ Our constructs, therefore, exhibit a decreased sensitivity to calcium *versus* normal cardiac tissue. This could be due to the lack for a well-organized tissue level architecture of the BEHMs, resulting in suboptimal calcium handling properties.

The responsiveness to isoproterenol also lends support to the functional organization of cardiac cells. Isoproterenol has been shown to result in a doubling of the active force production at a concentration of 30 μM in rabbit ventricular muscle.²⁴ In our BEHMs, we obtained a maximal response at a much lower isoproterenol concentration (50 nM), providing evidence of the presence of β -adrenergic receptors, an important physiological response of cardiac muscle.

Collectively, these performance metrics provide direct support for the functional organization of the cardiac cells into cardiac tissue in the BEHM.

The ability to electrically pace the construct shows some degree of cardiac phenotype and formation of functionally viable 3-dimensional heart muscle. The constructs were able to return to baseline force at the lower pacing frequencies of 1 and 2 Hz. However, at 5 and 7 Hz, we observed that the constructs were not able to return to baseline conditions. We believe that the reason for this is a lack of complete tissue level organization of cellular and extracellular components within the 3-dimensional architecture of the BEHMs. This potentially results in slower calcium transients, thereby limiting the number of active contractions per unit time, possibly due to a lower expression of SERCA.

We believe that the current model adds value to the field of cardiac tissue engineering by presenting a novel way to engineer 3-dimensional heart muscle *in vitro*. Fibrin has several characteristics that make this suitable to support the formation of cardiac patches and we believe that this works adds to the current literature on cardiac tissue engineering. Our current work demonstrates that cardiac patches generated utilizing fibrin have contractile performance

comparable to other published models of 3-dimensional heart muscle *in vitro*.

References

1. Chapekar MS. Tissue engineering: Challenges and opportunities. *J Biomed Mater Res* 2000;53:617–620.
2. Fuchs JR, Nasser BA, Vacanti JP. Tissue engineering: A 21st century solution to surgical reconstruction. *Ann Thorac Surg* 2001;72:577–591.
3. Lysaght MJ, Reyes J. The growth of tissue engineering. *Tissue Eng* 2001;7:485–493.
4. Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjak-Novakovic G. Cardiac tissue engineering: Cell seeding, cultivation parameters, and tissue construct characterization. *Biotechnol Bioeng* 1999;64:580–589.
5. Leor J, Abouafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium? *Circulation* 2000;102:56–61.
6. Li RK, Yau TM, Weisel RD, Mickle DA, Sakai T, Choi A, Jia ZQ. Construction of a bioengineered cardiac graft. *J Thorac Cardiovasc Surg* 2000;119:368–375.
7. Shapiro L, Cohen S. Novel alginate sponges for cell culture and transplantation. *Biomaterials* 1997;18:583–590.
8. Baar K, Birla R, Boluyt MO, Borschel GH, Arruda EM, Dennis RG. Heart muscle by design: Self-organization of rat cardiac cells into contractile 3-D cardiac tissue. *FASEB J* 2005;19:275–277.
9. Eschenhagen T, Fink C, Remmers U, Scholz H, Wattochow J, Weil J, Zimmermann W, Dohmen HH, Schafer H, Bishopric N, Wakatsuki T, Elson EL. Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: A new heart muscle model system. *FASEB J* 1997;11:683–694.
10. Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnol Bioeng* 2000;68:106–114.
11. Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(*N*-isopropylacrylamide). *J Biomed Mater Res* 1993;27:1243–1251.
12. Okano T, Yamada N, Okuhara M, Sakai H, Sakurai Y. Mechanism of cell detachment from temperature-modulated, hydrophilic–hydrophobic polymer surfaces. *Biomaterials* 1995;16:297–303.
13. Shimizu T, Yamato M, Kikuchi A, Okano T. Two-dimensional manipulation of cardiac myocyte sheets utilizing temperature-responsive culture dishes augments the pulsatile amplitude. *Tissue Eng* 2001;7:141–151.
14. Shimizu T, Yamato M, Akutsu T, Shibata T, Isoi Y, Kikuchi A, Umezu M, Okano T. Electrically communicating three-dimensional cardiac tissue mimic fabricated by layered cultured cardiomyocyte sheets. *J Biomed Mater Res* 2002;60:110–117.
15. Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ Res* 2002;90:e40.
16. Shimizu T, Yamato M, Kikuchi A, Okano T. Cell sheet engineering for myocardial tissue reconstruction. *Biomaterials* 2003;24:2309–2316.
17. Huang YC, Dennis RG, Larkin L, Baar K. Rapid formation of functional muscle *in vitro* using fibrin gels. *J Appl Physiol* 2005;98:706–713.

18. Boluyt MO, Zheng JS, Younes A, Long X, O'Neill L, Silverman H, Lakatta EG, Crow MT. Rapamycin inhibits α 1-adrenergic receptor-stimulated cardiac myocyte hypertrophy but not activation of hypertrophy-associated genes. Evidence for involvement of p70 S6 kinase. *Circ Res* 1997;81:176–186.
19. Dennis RG, Kosnik PE. Excitability and isometric contractile properties of mammalian skeletal muscle constructs engineered in vitro. *In Vitro Cell Dev Biol Anim* 2000;36:327–335.
20. Zimmermann WH, Schneiderbanger K, Schubert P, Didie M, Munzel F, Heubach JF, Kostin S, Neuhuber WL, Eschenhagen T. Tissue engineering of a differentiated cardiac muscle construct. *Circ Res* 2002;90:223–230.
21. Dar A, Shachar M, Leor J, Cohen S. Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. *Biotechnol Bioeng* 2002;80:305–312.
22. Layland J, Young IS, Altringham JD. The effect of cycle frequency on the power output of rat papillary muscles in vitro. *J Exp Biol* 1995;198:1035–1043.
23. Bers DM. *Excitation–Contraction Coupling and Cardiac Contractile Force*. Norwell:Kluwer; 2002. 427 p.
24. Endoh M, Blinks JR. Actions of sympathomimetic amines on the Ca^{2+} transients and contractions of rabbit myocardium: Reciprocal changes in myofibrillar responsiveness to Ca^{2+} mediated through α - and β -adrenoceptors. *Circ Res* 1988;62: 247–265.