Published in final edited form as:

ASAIO J. 2017 ; 63(3): 333–341. doi:10.1097/MAT.0000000000000486.

Electrical Stimulation of Artificial Heart Muscle: a look into the electrophysiological and genetic implications

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Abstract

Development of tissue-engineered hearts for treatment of myocardial infarction or biological pacemakers has been hindered by the production of mostly arrhythmic or in-synergistic constructs. Electrical stimulation (ES) of these constructs has been shown to produce tissues with greater twitch force and better adrenergic response. In order to further our understanding of the mechanisms underlying the effect of ES, we fabricated a bioreactor capable of delivering continuous or intermittent waveforms of various types to multiple constructs simultaneously. In this study, we examined the effect of an intermittent biphasic square wave on our artificial heart muscle (AHM) composed of neonatal rat cardiac cells and fibrin gel. Twitch forces, spontaneous contraction rates, biopotentials, gene expression profiles, and histological observations were examined for the ES protocol over a 12 day culture period. We demonstrate improved consistency between samples for twitch force and contraction rate, and higher normalized twitch force amplitudes for electrically stimulated AHM. Improvements in electrophysiology within the AHM was noted by higher conduction velocities and lower latency in electrical response for electrically stimulated AHM. Genes expressing key electrophysiological and structural markers peaked at days 6 and 8 of culture, only a few days after the initiation of ES. These results may be used for optimization strategies to establish protocols for producing AHM capable of replacing damaged heart tissue in either a contractile or electrophysiological capacity. Optimized AHM can lead to alternative treatments to heart failure and alleviate the limited donor supply crisis.

Keywords

Cardiac tissue engineering; biological pacemakers; heart muscle; electrical stimulation; electrophysiology

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Conflict of Interest

The authors declare no conflict of interest.

Statement of Human Studies

No human studies were carried out by the authors for this article.

Statement of Animal Studies

Approval for animal use was granted by the Institutional Animal Care and Use Committee (IACUC) at the University of Houston, in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, 1996).

1 Introduction

Development of a curative treatment for heart failure may be one of the paramount health care discoveries of our lifetime. Nearly 84 million people in the US have some form of cardiovascular disease, and nearly 1 in 9 deaths are attributable to complete heart failure [1]. Although heart transplant is the leading treatment for total heart failure, only about half of surgeries performed lead to a 10 year survival rate [2]. Heart failure related to disorders in cardiac rhythm resulted in the implantation of nearly 3 million permanent pacemakers between 1993 and 2009 [3]. Exacerbating the social implications is that the cost of the procedure increased by 45.3% over the same time span [3]. Unfortunately, this cost increase did not equivalently translate to improved survival statistics [4]. Survival rates are presently at 50% for non-severe cases, and even lower for patients with atrial fibrillation or complete heart block [4].

Finding alternative treatments that are both affordable and maintain desirable longevity are challenging. Yet hope remains as newly emerging technology in the field of tissue engineering continues to develop. By definition, tissue engineering is the development of biological constructs, composed of cells and a scaffold, capable of mimicking the function of natural tissues. By utilizing bioreactors, stimuli of various forms can be used to tune and enhance the functionality of the constructs, as well as encourage the maturation of cells. In particular, electrical stimulation (ES) bioreactors have been implemented in the hopes of improving tissue-engineered cardiac constructs for various heart failure treatments.

Recently, our lab developed fibrin gel based artificial heart muscle (AHM) capable of contracting on average at a twitch force of $\sim 4\text{mN/mm}^2$. These results mimic $\sim 10\%$ of the contractile force generated by isolated cardiac segments [5], [6]. It is important to note that, our developed constructs spontaneously contract, yet not at consistently rhythmic rates. We identify this as a notable sign of immaturity [7] and the presence of competing pacemaker cells. Thus, we have implemented ES bioreactors to promote rhythmic contraction. Our goal is to also enhance and retain function during culture, which typically degrades over time. As stated in previous publications, ES conditioning at low contractile rates may reduce the metabolic activity of the myocytes and improve the longevity of the constructs in culture [8]. The results of this and subsequent work may provide optimization strategies to produce either passive or active functional cardiac patches for mechanical or electrophysiological repair of a damaged heart. In particular, this work focuses on controlling and correcting the spontaneous arrhythmic contractions of our AHM and delves into the genetic implications of ES.

2 Materials and Methods

2.1 Isolation of Cardiac Cells

All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified. Cardiac cells were isolated from 2–3 day old neonatal Sprague-Dawley rat hearts using an established digestion method [9]. This isolation protocol obtains a mixture of cardiomyocyte, fibroblast, smooth muscle, endothelial, and cardiac stem cells, which are in

similar proportion to the natural heart composition. Cells from all the digests were pooled, centrifuged and then suspended in culture medium consisting of M199, 20% F12k, 10% fetal bovine serum, 5% horse serum, 1% antibiotic-antimycotic, 40 ng/ml of hydrocortisone, and 100 ng/ml of insulin.

2.2 Fabrication of Artificial Heart Muscle

Artificial heart muscle (AHM) was fabricated using a modified protocol from our lab [10]. Two AHM sizes were fabricated, depending on their use for contractile measurements or gene analysis. A mini-AHM for gene analysis was fabricated as a more economic and high-throughput alternative, while still maintaining similar functionality and physiology to the normal AHM. For the AHM fabricated for contractile properties analysis, fibrin gel was formed by polymerizing 10 units of thrombin in 1ml of culture medium with 10 units of fibrinogen in 500 μ l of saline in 35mm diameter culture dishes. For the mini-AHM fabricated for gene expression analysis, fibrin gel was formed by polymerizing 2.5 units of thrombin in 250 μ l of culture medium with 2.5 units of fibrinogen in 125 μ l of saline for each of the 16mm diameter wells of the ES bioreactor. Isolated cardiac cells were diluted in culture media at a density of 4×10^6 and 1×10^6 cells for regular and mini-AHM, respectively. For the AHM, minuten pins were placed at the 4 corners of a 2 $\times$ 2cm square template centered to a 35cm culture dish. For the mini-AHM, no minute pins were used and the mini-AHM was allowed to delaminate to the penetrating electrodes. The constructs were cultured in an incubator at 37°C and 5% CO₂ with culture medium changes every 2 days.

2.3 Electrical Stimulation (ES) of AHM

ES was applied to AHM through a custom built bioreactor capable of delivering stimulus to multiple AHM simultaneously, as shown in Figure 1. Three AHM are stimulated from a single channel output from a DAQ through 316 grade stainless steel electrodes spaced 5mm apart in each dish. The circuit contains trichromatic LEDs to indicate delivery of stimulus to each well, with color, green for cathodic and blue for anodic wave (red color unused), dictated by the direction of current allowed through the diodes. A 1k Ω resistor and a 10 μ F capacitor for each channel is added to further control the current and balance the charge delivered to the AHM.

For the mini-AHM (Figure 1B), the stainless steel electrodes pierced through from the bottom of the 16mm well and emerged in the medium. Preliminary studies showed that direct contact of the electrodes had a more profound effect on gene expression and contractile function. However, regular AHM (Figure 1A) for contractile measurements were not pierced with the electrodes to prevent structural damage during prolonged culture and transport from ES bioreactor to the twitch force and biopotential measurement systems. As such, the electrodes were placed adjacent to an edge of the regular AHM during culture, and the entire ES system was removed for physiological measurements.

Both AHM groups were stimulated with a biphasic square waveform. The output, illustrated in Figure 1C, was measured by an oscilloscope directly attached to the electrodes. In LABVIEW, two monophasic square waveforms of opposite polarity at + or – 2.5V, 3Hz, and 20% duty cycle were generated at an 80° phase shift and combined to form the biphasic

wave. At the electrode spacing of 5mm, the electric field strength would thus be 5V/cm. The reason for using a 3Hz waveform, higher than the conventional 1Hz, is to elevate cardiac function of the AHM closer to the native rat heart of ~300bpm, without exhausting the cardiac myocytes in an oxygen poor culture environment. Spontaneous contractions of our AHM, which are arrhythmic throughout culture, can range from less than 1Hz to 6Hz during contraction periods. The 80° phase shift induces an interphase delay between the anodic and cathodic waves which improves threshold for stimulation and reduces the accumulation of deleterious Faradic reaction products [11].

The generated electrical stimulus translates to a charge balanced biphasic wave with an interphase delay of ~10ms. The stimulus was intermittently delivered to the AHM in 30min intervals, separated by 30min rests, to encourage retention of spontaneous contractions. Preliminary studies showed that continuous stimulation inhibited spontaneous contractions in the majority of ES AHM. A 30min rest time is also a means of further reducing the production of Faradic reaction products and electrode corrosion. ES was initiated at day 2 of culture and continued until 12 days of culture for regular AHM and 10 days for mini-AHM.

2.4 Contractile Properties Measurements

Starting on day 4 of culture, 2 days after initiation of ES, spontaneous twitch force was measured every 2 days. These measurements were performed with a high sensitivity isometric force transducer (MLT0202, ADInstruments, Colorado Springs, CO) connected to a quad bridge amplifier (FE224, ADInstrument). By day 4 of culture, the AHM has delaminated and compacted into a 2 × 2cm square patch. The contractile force was measured by attaching the force transducer arm through a steel claw to one free-corner of the square patch, while the other three ends were held fixed. Spontaneous contractions were recorded for 30–60 seconds on both control and electrically stimulated AHM. LabChart 8 (ADInstruments) was used for data analysis with the Peak Analysis module using the General – Unstimulated analysis setting and a minimum peak height of 1.5 standard deviations, to calculate a mean twitch force for each sample. For each AHM, twitch force measurements from days 6 through 12 were normalized to day 4, to observe individual twitch force changes over time and reduce the effect of inter- and intra-group variations. Spontaneous contractile rate, which was measured in quasi-real-time on a separate channel, was averaged for the time block per sample.

ES response properties for both ES and control AHM were measured by electrically stimulating using a 3V, 1Hz, 10ms pulse width, monophasic square waveform. The latency value of the evoked response contraction, the contraction following an electrical stimulus, was measured using LabChart Peak Analysis module using the Evoked Response analysis setting. In the absence of a response contraction, no latency value was tabulated for the particular impulse into the mean latency value calculation for the sample. A representative plot of twitch forces for ES and control AHM is depicted in Figure 2, where the pulse train of the 1Hz stimulation is marked in conjunction to the measured contractile twitch force.

2.5 Cardiac Biopotential Measurements

Assessment of cardiomyocyte cell-to-cell interactions was performed by measurement of cardiac biopotential. Control and electrically stimulated AHM that were cultured for 12 days, and measured for twitch force, contractile rate, and latency throughout, were then measured for biopotentials as an end point measure. Biopotential measurements were carried out using a 32-electrode, gold-plated, 4 by 8 array, biopotential sensor developed by our lab [12]. The raw data was scanned using a custom MATLAB script for segments of a periodic waveform in the majority of the channels, signifying a consistent depolarization wave throughout an AHM. Selected data was then analyzed using cross correlation to determine the lag and calculate the conduction velocities throughout an AHM.

2.6 Gene Expression

Mini-AHM were collected on day 4, 6, 8, and 10 of culture in triplicates for control and ES AHM. Collected samples were placed in TriPure and manually agitated to encourage cell lysis and RNA extraction and then immediately frozen at -80°C until RNA isolation. The samples were thawed and the remaining fibrin gel removed. RNA was isolated by chloroform extraction and ethanol precipitation. Reverse transcription was performed using VILO master mix (Thermo Scientific). Quantitative PCR was run using SYBR master mix with CAV3.1, CACNAC1c, SERCA2, CX45, SCN7a, NAV1.5, TNNT2, and JPH primers with GAPDH as housekeeping gene (primer sequences in Supplemental Table 1). Results for days 4 through 10 from both control and ES AHM groups were normalized to a baseline value measured for AHM at day 2, before initiation of ES.

2.7 Histological Assessment

AHM at day 12 were fixed in ice-cold acetone for 20min. The fixed AHM was cropped down to a 10mm by 10mm square section of the center region. Nonspecific epitope antigens were blocked with 10% goat serum for 1 hour at room temperature (RT). Sections were incubated for 2 hours at RT with specific mouse anti- α -actinin monoclonal antibody (Sigma, Catalog No A7811) 1:500 and rabbit anti-connexin 43 (Cx43) (Abcam, ab11370) 1:500. Subsequently, sections were treated with goat anti-mouse and goat anti-rabbit secondary antibodies (Alexa Fluor 488, Alexa Fluor 546, Life Technologies, Grand Island, NY) 1:1000 for 1 hour at RT. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (2.5 $\mu\text{g}/\text{ml}$) for 5 min at RT. Fluorescent multi-layer, z-stack, images were obtained with a Nikon C2+ confocal laser-scanning microscope (Nikon Instruments Inc., Melville, NY) over a $635\mu\text{m}^2$ field of view and $10\mu\text{m}$ in depth. Signal volume ratios were calculated from signal volume measurements taken using Nikon C software and the z-stacks on a fixed threshold, relative to the total volume of the measured signals and the total volume of the field of view.

3 Results

3.1 Contractile Properties and Electrical Response

Consistent twitch force output and rhythmic contraction is the staple of an optimized AHM. The effect of ES on AHM twitch force was observable in direct functional improvement. As shown in Figure 3, on the 6th day of culture, 4 days after initiation of ES, electrically

stimulated AHM had a significantly greater twitch force increase than unstimulated control AHM (<2 fold difference). This difference grew throughout culture up to day 10, where it was over 3.3 fold greater for electrically stimulated AHM. A reduction of this improvement occurred at day 12, yet electrically stimulated AHM were still higher compared to control AHM. In general, ES did not have a significant impact on the maximum twitch force an AHM is capable of generating. The average spontaneous twitch force throughout culture was $4.28 \pm 2.12 \mu\text{N}/\text{mm}^3$ (n=10) for control and $4.49 \pm 1.64 \mu\text{N}/\text{mm}^3$ (n=18) for electrically stimulated AHM.

Electrically stimulated AHM was paced at a rate approximately half of a healthy resting rat's heart rate, ~360bpm [13]. This enabled us to more clearly determine if electrically stimulated AHM adapts to a pacing frequency during culture compared to the unstimulated control. The effect of ES on AHM contraction rate is even more profound. Electrically stimulated AHM contracted at rates that more closely hovered around the ES rate of 3 Hz, and with lower intragroup variation. The mean contraction rate was $291.7 \pm 142.1\text{bpm}$ (n=10) and $173.0 \pm 63.2\text{bpm}$ (n=18) throughout culture for control and electrically stimulated AHM, respectively. This indicates adaptation of the electrically stimulated AHM to the 3Hz pacing frequency.

The electrically stimulated AHM also had a significantly faster and more consistent response to a 1Hz pacing frequency during twitch force measurements. When stimulating the samples at 1Hz, the latency was $164.0 \pm 38.2\text{ms}$ (n=10) and $100.1 \pm 5.4\text{ms}$ (n=18) for control and electrically stimulated AHM, respectively (Figure 4D). Lower latency values imply improved cell junction coupling and conductivity of the AHM.

3.2 Cardiac Biopotential

To further evaluate conductivity and efficacy of ES on improving electrophysiology of the AHM, we calculated the conduction velocity using a custom 32-electrode biopotential measurement system (Figure 4A–C). By averaging the conduction velocity throughout the AHM, an assessment of the electrophysiological state of the AHM was made. The conduction velocities on average were $154 \pm 102\text{cm/s}$ (n=10) and $424 \pm 138\text{cm/s}$ (n=18) for control and electrically stimulated AHM, respectively (Figure 4E). The faster conduction velocities for electrically stimulated AHM correlates to the lower latency values obtained for the contractile response above. Both of these values signify more mature and well-developed electrophysiological state for electrically stimulated AHM.

3.3 Gene Expression

Relative gene expression, illustrated in Figure 5 showed a peak in the range of day 6 or 8 post-cell plating for the majority of the chosen genes, 4 and 6 days after initiating ES. Further on, expression showed a reduction close to initial baseline values or slightly lower. The most dramatic change during the day 8 peak occurred for NAV1.5 and SCN7a, two voltage gated sodium channels. The lowest effect was observed in levels of TNNT2, with a slight increase on day 6 and then a decrease to half of baseline levels by day 8. The only gene that immediately exhibited diminishing expression CAV3.1, a T type low voltage activated calcium channel.

3.4 Histological Assessment

Qualitatively, ES AHM segments fixed at day 12 and stained with connexin45 (cx45) and α -actinin showed higher fluorescence levels overall and improved sarcomere alignment and organization (Figure 6). Quantifying this representation using relative signal volume indexes, as a ratio of the total measured signal from all fluorescence, showed higher expression of the key cardiac markers for ES AHM as compared to the unstimulated controls. Measuring these ratios as the total volume of the observed field of view also indirectly showed increased cell presence, as evident by the higher DAPI volume index.

4 Discussion

Previously, ES of pure myocyte cultures on monolayer and in constructs has been shown to have a direct impact on function and physiology. Our results showed that, ES of AHM, which is composed of a mixed cardiac cell culture, resulted in significant improvement of normalized twitch force according to the parameters outlined in this study. This improvement started within 2 days of initiating the stimulus, and maintained for at most 6 days (Figure 3). Individually, however, unstimulated control AHM were capable of spontaneous contractions that exceed the twitch forces of electrically stimulated AHM, particularly early on in culture. This is expected, since, ES may not induce myocyte proliferation, but it may activate more myocytes to contract in unison more regularly. A more rhythmic and synchronous contractile AHM may induce positive genetic implications that result in a more optimized heart construct for tissue engineering research.

ES of mixed cardiac cell cultures may also facilitate adaptation to a prescribed external pulse rate and improve conduction velocity. These electrophysiology characteristics are vital for the maturation of the AHM and for its potential as an in vivo support or repair construct for dysfunctional heart segments. The adaptation to the 3Hz ES rate is evidenced by the improved consistency in measured contraction rate to 180bpm of the electrically stimulated AHM over the control AHM. A one-way ANOVA comparing the results of control and electrically stimulated AHM showed a p-value of 0.5351 for twitch force and 0.1261 for contraction rate. Although neither values showed a significant difference between the ES and control groups, the lower p-value for contraction rate indicated that ES may have had more of an effect on contraction rate than on twitch force during the relatively short application period. A longer time frame may be necessary to achieve a significant difference in contraction rates and perhaps even twitch forces [14].

Improved conduction for electrically stimulated AHM was proven by two means in our study. First, we measured the mean latency in the time required by the AHM to exhibit an evoked response to an external stimulus. The latency values were significantly lower for the electrically stimulated AHM, which was $100.1 \pm 5.4\text{ms}$ compared to the control at $164.0 \pm 38.2\text{ms}$ throughout culture (p-value=0.003). The difference in latency values arose from the speed of the depolarization wave initiated by the electrodes and propagation across the AHM. This propagation speed seemed to be hindered for the control group. Second, the results from latency values were reinforced by direct measures of conduction velocity at different points on the AHM using our custom 32-electrode biopotential system. Electrically stimulated AHM had nearly 3 times higher mean conduction velocity than control AHM, at

~4m/s (Figure 4). This is an interesting result since natural conduction velocities within the whole heart range from 3–5m/s in Purkinje fibers to 0.5-1m/s in working ventricular myocytes (15). ES, as such, may have improved the development of a Purkinje fiber-like system within the AHM, which resulted in the measured higher conduction velocity and lower latency.

In order to further understand the mechanism of AHM response to external electrical stimulation, we began by selecting gene targets that regulate electrophysiological components, i.e. membrane or sarcoplasmic ion channels and cell-cell interaction regulators. A striking phenomenon in our constructs was that the majority of the significant gene changes occurred at 4 or 6 days after the initiation of ES, as shown in Figure 5. The genes that showed the most significant elevations at that time were Nav1.5 and SCN7a, which both code for voltage gated sodium channels. There was a strong correlation between elevated expression levels of signal transduction pathways and voltage gated ion channels and ES, and our results supported those findings [16]. The upregulation of CX45, a cardiac specific gap junction protein, was also significantly evident on day 6, and the histological assessment reinforces the translation of the CX45 sequences into functional protein.

The drop in expression levels for the majority of the genes at day 10 may be a result of fibroblast overgrowth. Since fibroblast proliferation is not controlled in our protocol, and myocyte proliferation is limited to a single division, if any, the effect on gene expression may be diluted by fibroblast RNA. The chosen genes should not be actively expressed in fibroblasts, and, likewise, may not be upregulated by ES. The drop in expression levels may also correlate to the drop in twitch force for both control and ES AHM at day 12. Further evaluation of these findings by implementing strategies for controlling fibroblast growth may shed light on this mystery.

To provide insight into the effects of ES on the expression of genes coding for structural and contractile proteins, we selected TNNT2, a marker for troponin T type 2. The results showed a two-fold increase in expression at day 6. We hypothesized that coupling electrical and mechanical stimulation, by applying an oscillating load, may more directly enhance the expression of TNNT2, as well as other structural and contractile proteins. This was evident in the histological assessment of the α -actinin marker, which shows the resulting improved sarcomere organization and alignment relative to control AHM (Figure 6). The higher α -actinin signal volume index ratio for ES AHM reinforced this qualitative evaluation.

Recently published work by Hirt et al applied an ES protocol of 2V-4ms biphasic square pulses, having 2ms in each polarity with no interphase delay, on rat and human heart muscle constructs. This protocol was applied for a maximum pacing duration of 32 days [14]. Twitch forces for human heart muscle constructs stimulated at 1.5Hz surpassed unstimulated controls after 4–5days, and after 10 days reached ~50% higher forces to ~0.08mN. Rat constructs reached their maximum twitch force after three weeks, which was double the amplitude of unstimulated controls. Gene analysis identified a single rise in expression of potassium voltage-gated channel subfamily J member 8 (KCNJ8) [17], a membrane potential-stabilizing potassium current protein, along with an increase in markers for inflammatory response. Another study, from Blinova et al, showed that applying an

electrical field stimulus of 4 separate 5 ms biphasic square waves with a 30ms interphase delay during the refractory period increases intracellular calcium concentration by ~30% and length shortening by ~35% for isolated single cell measurements [18]. Left ventricular pressure in whole rat hearts was also observed to increase by ~60%, which was negated in the presence of a β 1-adrenoreceptor antagonist.

Previous studies have shown that ES may increase contractile protein content and improve alignment, but the mechanism by which this occurs is unclear. ES may directly modulate gene expression, or it may operate indirectly via a mechanotransduction cascade activated by increased contraction [19], [20]. What is clear is that ventricular myocytes that do not spontaneously contract, either inherently or by neighboring pacing cells, may now add to the functionality of a cardiac construct. An active myocyte maintains its phenotype longer in culture, whereas an unstimulated myocyte has the tendency to undergo a dedifferentiation process in vitro [21]. These studies, along with others [19], [22], demonstrated improvement in heart tissue function by increased contractile function, histological assessments, and a few key gene upregulations. Yet, more evaluation of the electrophysiological impact of ES on engineered heart tissue must still be performed to validate its use as a reparative treatment for heart failure.

5 Conclusion

Electrically stimulation has been shown to improve function of tissue engineered cardiac constructs, which our results along with others reinforce, but little has been done to understand the mechanisms underlining this effect. We have performed an initial report on the physiological and gene expression modifications that occur for artificial heart muscle electrically stimulated with a biphasic waveform. ES has significantly improved twitch forces, conduction velocities, and latency values and upregulated key cardiac markers that improve both the structural and electrophysiological functionality of the cardiac constructs. Further evaluation of the pathways involved, whether by direct electrical induction or an indirect mechanotransduction cascade, may provide valuable insight in the development and optimization of artificial heart muscle for mechanical and electrophysiological support of failing hearts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The researchers would like to acknowledge NIH for provision of funding for this research (Grant number: R01-EB011516). We would also like to thank the Department of Biomedical Engineering and the Cullen College of Engineering at University of Houston for further financial support. We also greatly appreciate the help from Dr. Keri Schadler for editing this manuscript.

Source of Funding

The research was funded by an NIH R01 awarded to RKB, grant number: R01-EB011516.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart Disease and Stroke Statistics – 2014 Update: A report from the American Heart Association. 2014; 129(3)
2. Mastrobuoni S, Dell'Aquila AM, Azcarate PM, Rabago G, Herreros J. Long-term survival (>20 years) following heart transplantation. *J Cardiovasc Surg (Torino)*. Oct; 2012 53(5):677–84.
3. Greenspon AJ, Patel JD, Lau E, Ochoa JA, Frisch DR, Ho RT, Pavri BB, Kurtz SM. Trends in permanent pacemaker implantation in the United States from 1993 to 2009: Increasing complexity of patients and procedures. *J Am Coll Cardiol*. 2012; 60(16):1540–1545. [PubMed: 22999727]
4. ALT E, VOLKER R, WIRTZFELD A, ULM K. Survival and Follow-up After Pacemaker Implantation: A Comparison of Patients With Sick Sinus Syndrome, Complete Heart Block, and Atrial Fibrillation. *Pacing Clin Electrophysiol*. Nov; 1985 8(6):849–855. [PubMed: 2415938]
5. Tao Z-W, Mohamed M, Hogan M, Gutierrez L, Birla RK. Optimizing a spontaneously contracting heart tissue patch with rat neonatal cardiac cells on fibrin gel. *J Tissue Eng Regen Med*. Apr.2014
6. Milani-Nejad N, Brunello L, Gyorke S, Janssen PML. Decrease in sarcoplasmic reticulum calcium content, not myofilament function, contributes to muscle twitch force decline in isolated cardiac trabeculae. *J Muscle Res Cell Motil*. Jul; 2014 35(3–4):225–234. [PubMed: 25056841]
7. Jacobson S, Piper H. Cell cultures of adult cardiomyocytes as models of the myocardium. *J Mol Cell Cardiol*. Jul; 1986 18(7):661–678. [PubMed: 3528506]
8. Hirt MN, Hansen A, Eschenhagen T. Cardiac tissue engineering: state of the art. *Circ Res*. 2014; 114(2):354–367. [PubMed: 24436431]
9. Huang Y-C, Khait L, Birla RK. Contractile three-dimensional bioengineered heart muscle for myocardial regeneration. *J Biomed Mater Res A*. Mar; 2007 80(3):719–31. [PubMed: 17154158]
10. Tao Z-W, Mohamed M, Hogan M, Gutierrez L, Birla RK. Optimizing a spontaneously contracting heart tissue patch with rat neonatal cardiac cells on fibrin gel. *J Tissue Eng Regen Med*. Apr.2014
11. Merrill DR, Bikson M, Jefferys JGR. Electrical stimulation of excitable tissue: Design of efficacious and safe protocols. *J Neurosci Methods*. 2005; 141(2):171–198. [PubMed: 15661300]
12. Salazar B, Reddy A, Tao Z, Madala S, Birla R. 32-Channel System to Measure the Electrophysiological Properties of Bioengineered Cardiac Muscle. *IEEE Trans Biomed Eng*. Feb. 2015
13. Azar T, Sharp J, Lawson D. Heart rates of male and female Sprague-Dawley and spontaneously hypertensive rats housed singly or in groups. *J Am Assoc Lab Anim Sci*. Mar; 2011 50(2):175–84. [PubMed: 21439210]
14. Hirt MN, Boeddinghaus J, Mitchell A, Schaaf S, Börnchen C, Müller C, Schulz H, Hubner N, Stenzig J, Stoeck A, Neuber C, Eder A, Luther PK, Hansen A, Eschenhagen T. Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation. *J Mol Cell Cardiol*. 2014; 74:151–161. [PubMed: 24852842]
15. Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *American Journal of Physiology*. 1999; 277(2:Pt 2):433–444.
16. Xu J, Wang W, Clark CC, Brighton CT. Signal transduction in electrically stimulated articular chondrocytes involves translocation of extracellular calcium through voltage-gated channels. *Osteoarthritis Cartil*. 2009; 17(3):397–405. [PubMed: 18993082]
17. Zhang H, Flagg TP, Nichols CG. Cardiac sarcolemmal KATP channels: Latest twists in a questing tale! *J Mol Cell Cardiol*. 2010; 48(1):71–75. [PubMed: 19607836]
18. Blinova K, Stohlman J, Krauthamer V, Knapton a, Bloomquist E, Gray Ra. Acute effects of nonexcitatory electrical stimulation during systole in isolated cardiac myocytes and perfused heart. *Physiol Rep*. 2014; 2(8):e12106–e12106. [PubMed: 25096553]

19. Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proc Natl Acad Sci U S A*. 2004; 101(52):18129–18134. [PubMed: 15604141]
20. Hirt MN, Hansen A, Eschenhagen T. Cardiac tissue engineering: state of the art. *Circ Res*. Jan; 2014 114(2):354–67. [PubMed: 24436431]
21. Zhang Y, Li T-S, Lee S-T, Wawrowsky KA, Cheng K, Galang G, Malliaras K, Abraham MR, Wang C, Marbán E. Dedifferentiation and Proliferation of Mammalian Cardiomyocytes. *PLoS One*. 2010; 5(9):e12559. [PubMed: 20838637]
22. Nunes SS, Miklas JW, Liu J, Aschar-Sobbi R, Xiao Y, Zhang B, Jiang J, Massé S, Gagliardi M, Hsieh A, Thavandiran N, Laflamme Ma, Nanthakumar K, Gross GJ, Backx PH, Keller G, Radisic M. Biowire: a platform for maturation of human pluripotent stem cell-derived cardiomyocytes. *Nat Methods*. 2013; 10(8):781–7. [PubMed: 23793239]

Figure 1. Bioreactor and Waveform

Electrical stimulation bioreactor coated with PDMS and consisting of 2 or 4 separate channels for AHM and mini AHM samples, respectively. Each channel output from the DAQ stimulates samples in three wells through 316 grade stainless steel electrodes. The circuit contains trichromatic LEDs to indicate delivery of stimulus to each well, with color, green for cathodic and blue for anodic wave, dictated by the direction of current allowed through the diodes. A $1\text{k}\Omega$ resistor and a $10\mu\text{F}$ capacitor for each channel is added to further control the current and balance the charge delivered to the AHM. **A)** Two models of the electrical stimulation bioreactor for AHM used in physiology tests. **B)** Mini AHM bioreactor for gene expression tests. **C)** The LABVIEW output options result in the illustrated biphasic square wave measured with an oscilloscope at the electrodes while submerged in culture media. With a culture media resistance of $\sim 3\text{k}\Omega$, the transient current through the AHM is $\sim 40\mu\text{A}$.

Figure 2. Representative Twitch Force Plots

Twitch force was measured using an isometric force transducer attached to one of four AHM corners, while keeping the other three fixed (Left). Twitch force plots exported from PowerLab system as a representation of the typical responses from control and electrically stimulated AHM (Right). Control AHM typically have a more delayed response to the 1Hz electrical stimulation applied during electrical response tests. Latency is calculated as the time between the electrical stimulation pulse and the next corresponding twitch force peak on a superimposed plot.

Figure 3. Twitch Force and Contraction Rate

Spontaneous contractile output normalized to day 4 maintained a significantly higher increase for ES AHM on days 6, 8, and 10 (* $p < 0.05$). Spontaneous contractile rate hovered around the 3 Hz stimulation rate (180 BPM) for the ES AHM throughout culture, while control AHM maintained elevated rates closer to that of the natural rat heart. Number of samples used were $n=18$ for ES AHM and $n=10$ for control AHM.

Figure 4. Biopotential Conduction Map, Conduction Velocity, and Latency
(A) Biopotential measurement from a custom 32 electrode system visualized using MATLAB over a 3 sec timespan. (B) Maps of the lags output from cross correlation between the measured biopotentials in reference to each of the 32 electrodes, a measure of time of peak incidence in time. (C) Maps of the conduction velocities for each of the 32 electrodes with one as a reference. (D) Electrical response latency was 100.1 ± 5.4 ms and 164.0 ± 38.2 ms for electrically stimulated and control AHM, respectively. (E) The mean conduction velocities were 424 ± 138 cm/s and 154 ± 102 cm/s for electrically stimulated and control AHM, respectively.

Figure 5. PCR Relative Cardiac Gene Expression

Gene expression profiles of ES AHM (n=6 each time point) relative to control AHM (n=4 each time point) of select cardiac specific electrophysiological proteins and ion channels, values >1 signify expression higher than control AHM. Expression values were normalized to housekeeping gene GAPDH. Day 4 was a pivotal time in that it coincided with the activation of most of the genes examined as visible in the heat map.

Figure 6. Histological Assessment
Qualitative representation of the immunostained ES and control AHM depicts increased expression of key cardiac markers connexin45 (CX45, green) and α -actinin (red). The signal volume ratios show higher relative volume ratios of these key cardiac markers for ES AHM, and higher expression over the total volume of the observed field of view.