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Mimicking Isovolumic Contraction with Combined Electromechanical Stimulation Improves the Development of Engineered Cardiac Constructs

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Electrical and mechanical stimulation have both been used extensively to improve the function of cardiac engineered tissue as each of these stimuli is present in the physical environment during normal development *in vivo*. However, to date, there has been no direct comparison between electrical and mechanical stimulation and current published data are difficult to compare due to the different systems used to create the engineered cardiac tissue and the different measures of functionality studied as outcomes. The goals of this study were twofold. First, we sought to directly compare the effects of mechanical and electrical stimulation on engineered cardiac tissue. Second, we aimed to determine the importance of the timing of the two stimuli in relation to each other in combined electromechanical stimulation. We hypothesized that delaying electrical stimulation after the beginning of mechanical stimulation to mimic the biophysical environment present during isovolumic contraction would improve construct function by improving proteins responsible for cell–cell communication and contractility. To test this hypothesis, we created a bioreactor system that would allow us to electromechanically stimulate engineered tissue created from neonatal rat cardiac cells entrapped in fibrin gel during 2 weeks in culture. Contraction force was higher for all stimulation groups as compared with the static controls, with the delayed combined stimulation constructs having the highest forces. Mechanical stimulation alone displayed increased final cell numbers but there were no other differences between electrical and mechanical stimulation alone. Delayed combined stimulation resulted in an increase in SERCA2a and troponin T expression levels, which did not happen with synchronous combined stimulation, indicating that the timing of combined stimulation is important to maximize the beneficial effect. Increases in Akt protein expression levels suggest that the improvements are at least in part induced by hypertrophic growth. In summary, combined electromechanical stimulation can create engineered cardiac tissue with improved functional properties over electrical or mechanical stimulation alone, and the timing of the combined stimulation greatly influences its effects on engineered cardiac tissue.

Introduction

Cardiovascular disease is the leading cause of death in the United States[1] and is becoming one of the major causes of death worldwide.[2] The buildup of atherosclerotic plaques in the coronary arties leads to ischemia and, if untreated, results in a myocardial infarction (MI), the loss of contractile tissue, and subsequent reduction in heart function. Due to inadequate cardiomyocyte turnover, limited regenerative potential exists in the adult heart,[3] preventing the restoration of the lost contractile function. Though a heart transplant is the gold standard of care for heart failure patients,[4] there are limited number of hearts available and the patients require immunosuppressant therapies for the remainder of their lives.[5] Tissue engineering represents a novel treatment option for the repair and replacement of the infarcted myocardium.[6]

Native myocardium is a complex tissue, consisting of several types of cells that work cooperatively to ensure healthy heart function. For example, the cell–cell coupling of myocytes in the heart enables the propagation of electrical signals to produce the almost synchronous contractions of the whole organ to adequately pump blood to the body.[7] This excitation–contraction coupling of cardiac tissue is essential for the development and function of the organ, and recapitulating this response in engineered cardiac

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tissue could facilitate the development of more functionally appropriate constructs. As part of this approach, physical stimulation has been used to enhance the development of engineered myocardial constructs in order to generate engineered tissues with functional properties similar to native tissues. Zimmermann initially demonstrated that mechanical stimulation of tissue-engineered constructs improved cellular functionality by improving their organization and contraction force.[8] Cyclic stretch activates FAK and RhoA, which initiates myotube formation[9] and aids in extracellular matrix (ECM) formation.[10] Since this study, many groups have used stretch as a standard method of conditioning engineered myocardium *in vitro*.[11–15] Similarly, electrical stimulation has also been established as a method of stimulating constructs to induce synchronous contractions of engineered cardiac tissue. Radisic *et al.* initially demonstrated that electrical stimulation induces synchronous contractions, which enhances the conductive and contractile properties of the construct.[16] The general electrical field stimulation induces calcium transients in the cells, which improves the volume fraction and organization of sarcomeres in the tissue[17] and induces alignment of the cells,[18] promoting an increase in gap junction formation and localization.[19] Because of the improvements observed in construct development, electrical stimulation has been used in a number of other studies as a method of developing functional engineered cardiac constructs.[20–22]

Though both electrical and mechanical stimulation are standard methods of physical stimulation in cardiac construct development, to date, there have been no direct comparisons of the differences between electrical and mechanical stimulation. Attempting to compare published data is also difficult as these studies have been done in different systems with different measures of improvement. For example, the mechanical stimulation studies often looked at twitch force and related properties,[8,23,24] while electrical stimulation studies generally have assessed improvement through fractional area shortening, excitation threshold, and maximum capture rate.[22] These differences have inhibited the discussion of a comparison between electrical and mechanical stimulation, as well as any study on their interplay and the potential effects of a combined stimulation approach on the development of functional engineered heart tissue. Though bioreactors that have both electrical and mechanical stimulation capabilities have been created previously,[14,25,26] to date, only one study has looked into the effects of combined electromechanical stimulation in cardiac engineered tissue.[27] In this study, Wang *et al.* observed that electromechanical stimulation improved cardiac differentiation of mesenchymal stem cells and an increase in contractile proteins, such as α-actinin, myosin heavy chain, and connexin 43 (Cx43), as compared with those cultured statically. However, the electrical and mechanical stimulation were applied simultaneously, which is not physiologically relevant in the context of the heart, where mechanical stimulation represents the filling of the ventricles with blood and electrical stimulation of the ventricles to contract occurs just before the release of stretch.

During the cardiac cycle, there is a brief period of overlap between the mechanical stretch of the filled ventricle and the electrical stimulus that induces ventricular contraction. As the ventricle is stimulated to contract, it is maintained in the stretched state for a short period of time (called isovolumic contraction), until the pressure in the ventricle is high enough to cause the aortic valve to open. This overlap of the two stimuli results in a switching of the contractile mode of the muscle from isometric while the valve is closed ($\sim$10–30 ms[28]) to isotonic once the valve opens.[29] Under isometric conditions, similar to the isovolumic contraction, mechanical stretch shortens the intracellular calcium transient, which shortens the action potential duration.[30] During isotonic shortening, similar to the ejection phase of the cardiac cycle, the intracellular calcium transients are prolonged as is the action potential duration.[31] As such, it is likely that the switch in contractile modes could modulate the calcium-dependent effects on action potential duration,[29] which maximizes force generation and increases the speed of contraction and relaxation.[32]

We hypothesized that a delayed electrical stimulation after the beginning of the mechanical stretch that mimics the biophysical environment present during isovolumic contraction would improve the contractile performance of engineered cardiac constructs when compared with synchronized combined electromechanical stimulation or electrical or mechanical stimulation alone. To test this hypothesis, we created a novel bioreactor system that allowed for precise control of the timing of combined electrical and mechanical stimulation of engineered cardiac tissue constructs. The goals of this study were twofold. First, we wanted to directly compare the effects of electrical stimulation with mechanical stimulation. Second, we wanted to understand the effects of timing in combined electromechancial stimulation on construct development. Our data demonstrate that mechanical stimulation improved final cell numbers, but no other differences between the individual stimuli were observed. Further, we demonstrated that combined stimulated constructs with delayed electromechanical stimulation, similar to the stimuli observed during isovolumic contraction, improved calcium handling and contractile protein expression and led to the highest contractile force. In summary, the use of combined stimulation that mimicked the *in vivo* environment enhanced the function and maturation of engineered cardiac tissues over electrical or mechanical stimulation alone.

Materials and Methods

Cell isolation and culture

All animal experiments were performed in accordance with U.S. Animal Welfare Act and institutional guidelines and were approved by the Institutional Animal Care and Use Committee at Tufts University. Cardiomyocytes were isolated from 2- to 3-day-old neonatal Sprague–Dawley rats as previously described.[6,33] Briefly, hearts were isolated, minced to small pieces, and then digested with collagenase at 37°C. Freshly isolated cardiac cells were mixed into a fibrin-forming solution with a final concentration of 3.33 mg/mL bovine fibrinogen, 2 U/mL bovine thrombin, and 2 mM $CaCl_2$ as previously described.[6,33] This cell/fibrin solution was injection molded into tubular molds and allowed to incubate for 20 min at 37°C to allow the fibrin to polymerize (Fig. 1E). Constructs were cultured statically in jars containing culture medium (Dulbecco's modified Eagle's medium, 10% horse serum, 2% fetal bovine serum, 1% penicillin–streptomycin, 2 μM ε-aminocaproic acid, 2 μg/mL insulin, and ascorbic acid, 50 μg/mL) for 2 days before being transferred into a custom-made bioreactor for stimulation with the same media.

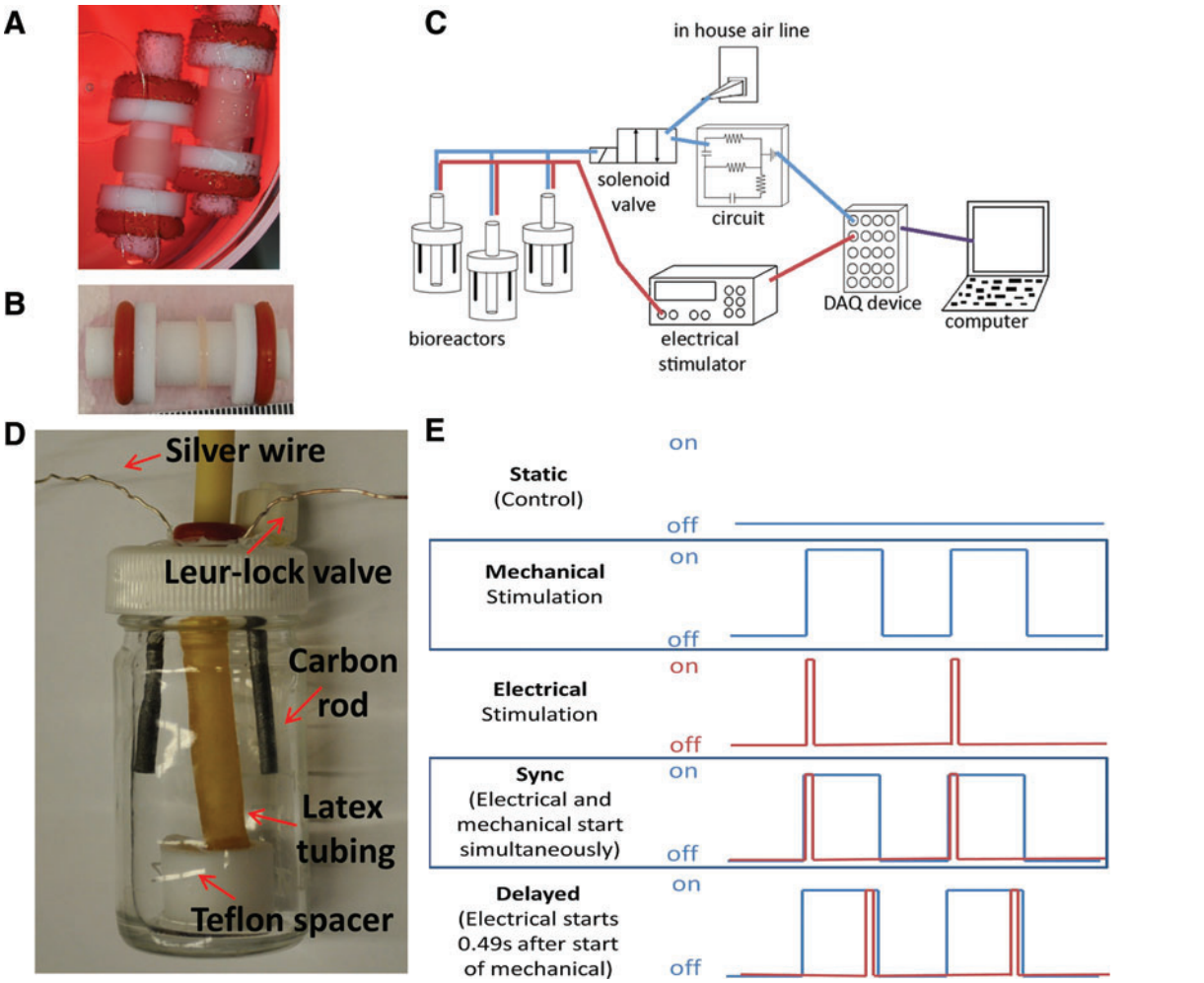

FIG. 1. Statically cultured constructs on day 1 **(A)** and day 14 **(B)**. **(C)** Schematic of the bioreactor system, showing the bioreactors connected to the solenoid valve, which is connected to the compressed air line in the lab. The carbon rods are connected to an electrical stimulator. The solenoid valve and the electrical stimulator are both controlled by the computer through a DAQ device. **(D)** An image of the custom distension bioreactor with individual components labeled. **(E)** A diagram demonstrating the stimulation regimens implemented. The blue line represents the mechanical stretch while the red line represents the electrical stimulation. Color images available online at www.liebertpub.com/tea

Bioreactor design

The bioreactor was based on a previously described design (Fig. 1B).[34] Briefly, the bioreactor contains a distensible mandrel that was created using a thin latex tubing (Kent Elastomer, Kent, OH) that expands when air is injected into the system and carbon rods on either side of the tube that induce the electrical stimulation. One end of the tube was closed with a Teflon stopper and epoxy sealant, while the other end was connected to an in-house air line regulated by a pressure regulator (Fig. 1A). When air is injected, the tubing expands creating a uniform distension that stretches the constructs circumferentially. The frequency and duty cycle of the air pressure were defined by the action of a solenoid valve connected downstream of the pressure regulator that was controlled by a custom-made LabVIEW algorithm via a National Instruments Data Acquisition Device (NI-DAQ) (National Instruments Corporation, Austin, TX) (Fig. 1D). The carbon rods were attached to the lid of the bioreactor and were connected in parallel with a custom circuit to an electrical muscle stimulator (Grass Technologies S88×, West Warwick, RI) that produced an electrical pulse when activated by the NI-DAQ. Numerous samples can be run with each condition, and multiple conditions can be run simultaneously (Fig. 1C). After 2 weeks of culture during which the constructs compact (Fig. 1B), each construct was harvested and analyzed for contraction force, before being divided into sections of equal length for DNA quantification, and protein extraction for western blots.

Stimulation regimes

Experiments were conducted to compare static culture, mechanical stimulation only, electrical stimulation only, synchronized electromechanical stimulation, and delayed electromechanical stimulation. Figure 1E shows a diagram that demonstrates the various conditions, where the blue line represents the mechanical stretch, while the red line represents the electrical pulse. Mechanical stimulation (Fig. 1E, second row) was carried out at 5% stretch with a 50% duty cycle at 1 Hz. Stimuli for electrical stimulation (Fig. 1E,

third row) were biphasic rectangular pulses, with a duration of 1 ms. The electrical stimulation was conducted at 1 Hz with a supra-threshold voltage of 3 V/cm. For the synchronized electromechanical stimulation (Fig. 1, fourth row), the electrical and mechanical stimulation began simultaneously. For the delayed electromechanical stimulation (Fig. 1, bottom row), the electrical stimulation began with 0.01 s remaining in the mechanical stimulation duty cycle to mimic overlap present in the isovolumic contraction.

Immunohistochemistry

A portion of each construct was fixed in 4% paraformaldehyde for 3 h at 4°C before being flash frozen in Tissue

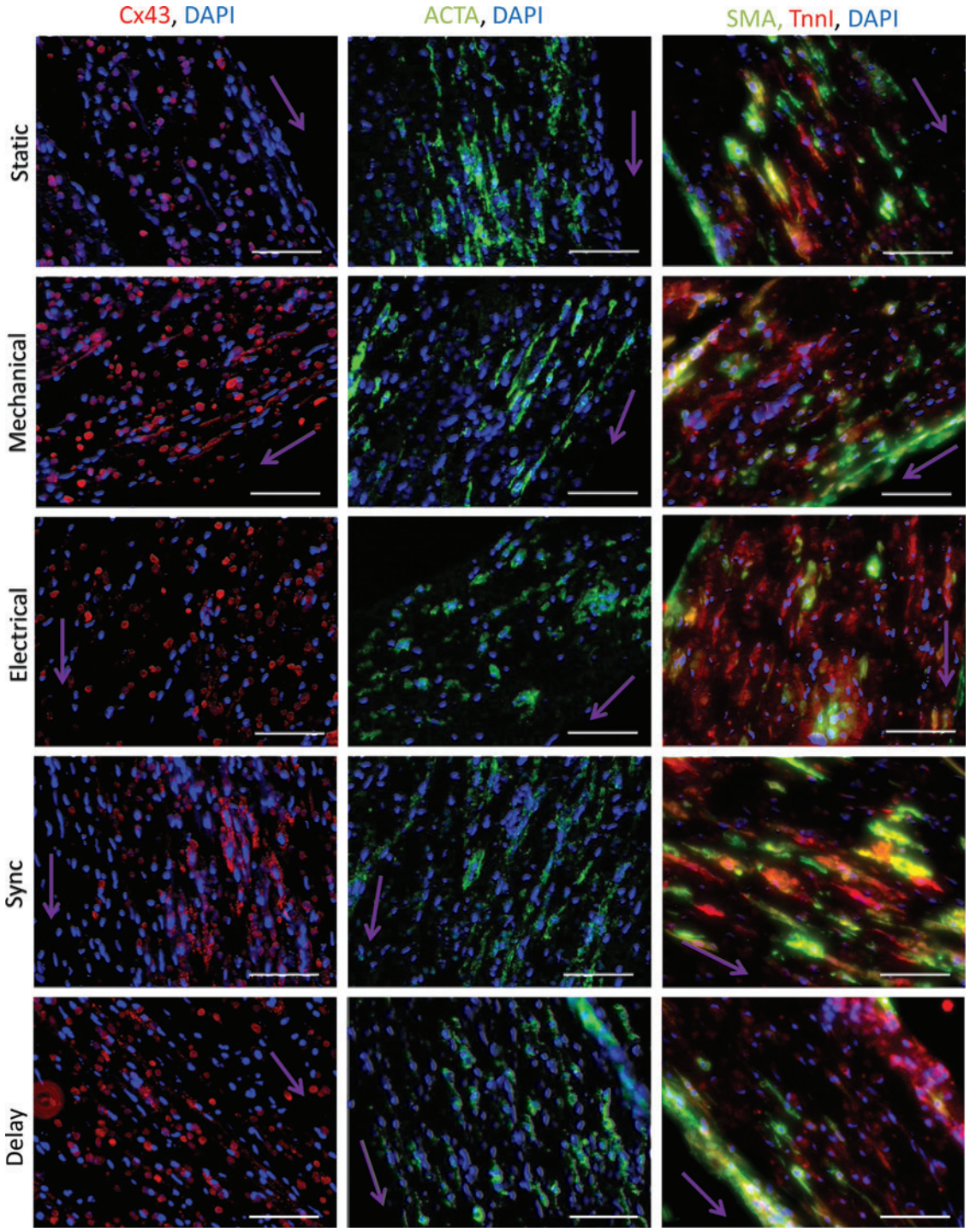

FIG. 2. Representative histology of constructs under each condition. Connexin 43 (Cx43) (red), cardiac α-actin (green), troponin I (red), and α-smooth muscle actin (green) are shown for all conditions. Cell nuclei are stained with DAPI. The purple arrow shows the direction of alignment and the circumferential axis of each construct (Scale bar = 100 μm). Color images available online at www.liebertpub.com/tea

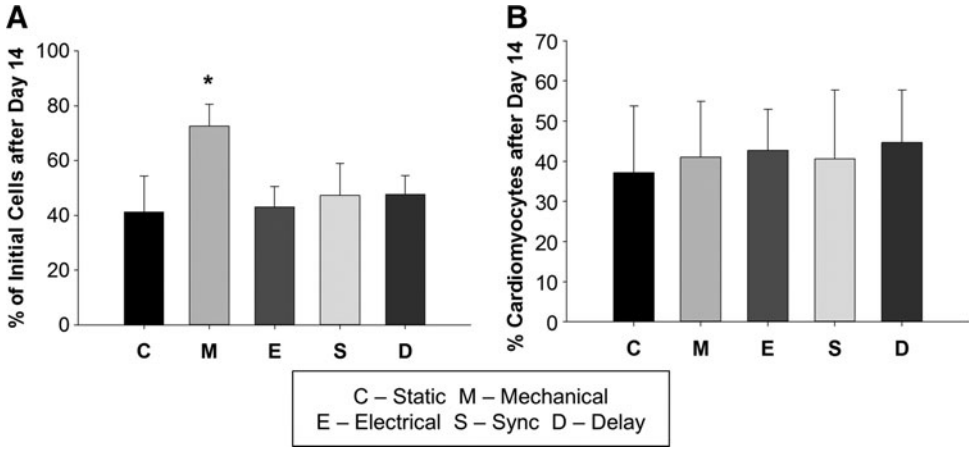

FIG. 3. **(A)** Percentage of initial cells in constructs after day 14 for constructs under statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). The symbol *denotes statistically different than all other conditions ($p < 0.05$). **(B)** Percentage of cardiomyocytes after day 14.

Tek O.C.T. freezing medium and sectioned into 10-μm slices. The sections were washed with phosphate-buffered saline (PBS), blocked with 5% donkey serum in PBS with 0.1% BSA, and stained labeled with antibodies against Cx43 (#3511S; Cell Signaling Technology, Danvers, MA), cardiac α-actin (sc-58670; Santa Cruz Biotechnology, Dallas, TX), and troponin I (cTnI) (#47003; Abcam, Cambridge, MA), for cardiomyocytes, and α-smooth muscle actin (α-SMA) (sc-32251; Santa Cruz Biotechnology), for fibroblasts (Fig. 2). Dilutions of the appropriate secondaries were used (Alexa Fluor 488-conjugated donkey anti-mouse A-21202 and Alexa Fluor 546-conjugated donkey anti-rabbit A10040; Invitrogen, Inc., Eugene, OR). Hoescht 33258 (H1938; Invitrogen, Inc.) at 0.1 μg/mL was used to counterstain DNA to label nuclei.

Percentage of cardiomyocytes

Immunofluorescent staining methods were similar to methods used in histology. Briefly, the sections were stained with troponin I for cardiomyocytes and α-SMA for fibroblasts. Eight fields per sample were necessary to image the entire section. The total number of cell nuclei, $cTnI^+$ cells, and $\alpha\text{-}SMA^+$ cells were determined using customized pipelines in CellProfiler (Broad Institute, Cambridge, MA). The percentage of cardiomyocytes was determined by taking the ratio of cardiomyocytes ($cTnI^+$ cells) to total number of cells ($cTnI^+$ cells and SMA^+ cells).

DNA quantification

DNA content was quantified with a modified Hoechst assay as previously described.[35] Briefly, a portion of each construct was digested in 0.5 mg/mL proteinase K in digestion buffer (100 mM tris and 50 mM EDTA, pH 7.4) overnight at 56°C. The assay was performed by combining 100 μL of the digested sample with 100 μL of the Hoechst dye solution (0.2 μg/mL Hoechst 33258, 2 M NaCl, 10 mM tris, and 1 mM EDTA, pH 7.4) per well in a 96-well black, clear-bottomed plate. The plate was read in a microplate reader (Bio-Tek Instruments, Inc., Houston, TX) at 360/460 nm excitation/emission. Calf thymus DNA in the digestion buffer at concentrations of 0–6000 ng/mL was used as standards. The cell concentrations, calculated as the total number of cells per unit volume, were obtained from DNA content by assuming 7.6 pg of DNA per cell.[36] Total cell number per construct (Fig. 3) was calculated for each sample by knowing the percentage of the construct used in the DNA assay based on image analysis of the construct portions for the assay and knowing the amount of cells in the digested sample. Briefly, for the image analysis, the sectioned construct is imaged and the total area of each section is determined in ImageJ. The amount of DNA from the portion of the construct used for DNA assay is normalized by the percent of the construct used for DNA analysis in order to reconstruct the total DNA count for the whole

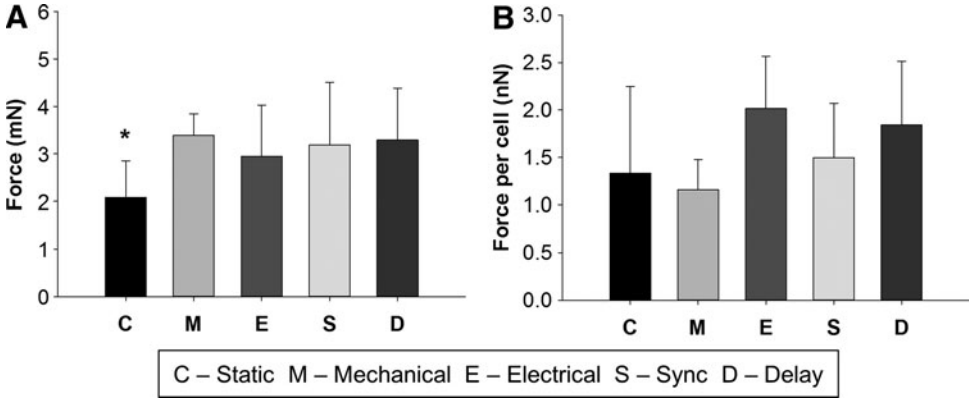

FIG. 4. **(A)** Observed twitch forces for all groups: statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). **(B)** Calculated twitch force per cell (nN). *denotes statistical significance of ($p < 0.05$).

construct. To understand the extent of cell loss upon construct construction, we calculated an initial cell number as the number of cells in the construct at day 0, 2 h after construct creation. The % of cells after day 14 was then determined to be the final cell number per construct divided by average initial cell number.

Twitch force measurements

Twitch force was measured using a custom-built testing system based on one previously described.[33] The constructs were paced using general field stimulation with a cardiac stimulator (S88×; Astro-Med, Inc., West Warwick, RI) connected to carbon rod electrodes in a bath. The twitch force was recorded with a preload of 10 mN in response to a 0.5-Hz pulse train of 6-ms long, monopolar square waves with amplitudes of 6 V/cm. Data were acquired using a custom-made LabVIEW algorithm (National Instruments Corporation). Briefly, the LabVIEW algorithm reads and records the twitch force measurements, which are outputted by the testing system through an NI-DAQ. The data are then analyzed offline using MATLAB (The Mathworks, Inc., Natick, MA). The twitch force was defined as the force observed from the baseline to the maximum height of the contraction following stimulation (Fig. 4A). The force per cell was defined as the force observed divided by the number of cells observed at day 14 (Fig. 4B). Contraction time was defined as the time for the construct to contract completely, and the contraction rate was calculated by dividing the twitch force by the time of contraction (Fig. 5). Relaxation time was defined as the time for the construct to relax to half of the observed twitch force and the relaxation rate was calculated by dividing half of the peak twitch force by the time of relaxation.

Western blotting

Western blotting for protein expression was carried out as previously described.[33] Tissues were rinsed with PBS and then disrupted by sonication in ice-cold lysis buffer containing protease inhibitors (25 mM Tris [pH 7.4], 225 mM sodium chloride, 5% glycerol, 0.5% Nonidet P-40, 0.025% sodium deoxycholate, 1 mM EDTA, 2 mM sodium orthovanadate, 1 μg/mL aprotinin, pepstatin, and leupeptin; Sigma-Aldrich, St. Louis, MO). The lysate was separated from the nonsolubilized matrix through centrifugation, and the protein

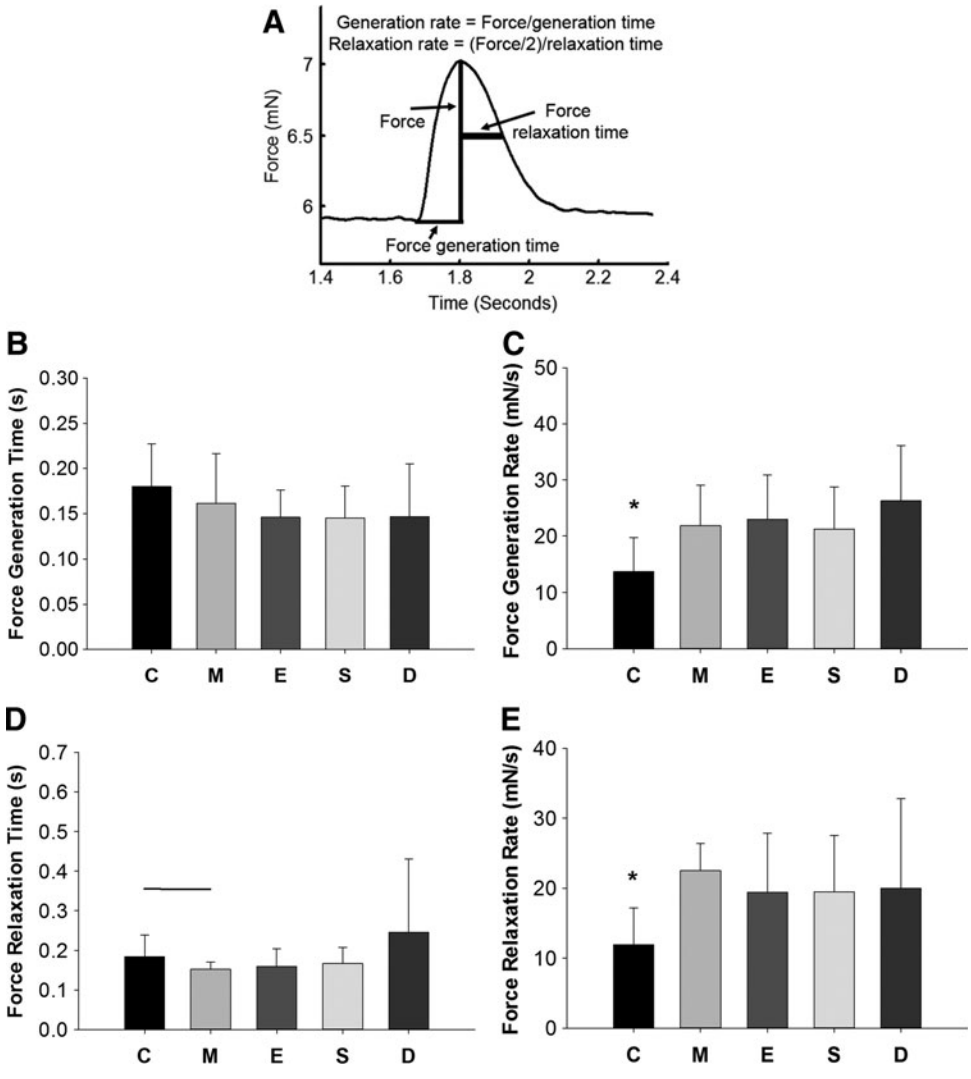

FIG. 5. **(A)** Example of twitch force profile that indicates the definition of twitch force, contraction time, relaxation time, and the corresponding rates. **(B)** Force generation time (s) for all conditions. **(C)** Force generation rate for all conditions (mN/s). **(D)** Force relaxation time (s) and **(E)** force relaxation rate for all conditions (mN/s). Conditions include statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). For all figures **(B–E)**, the lines show statistical significance of ($p < 0.05$) and *denotes statistically different than all other conditions ($p < 0.05$).

concentration was determined by BCA assay. Western blot analysis was conducted on $n=4$ for all groups. The lanes of the 4%–15% precast polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA) were loaded with 15 μg of protein and run before being transferred to a nitrocellulose membrane. A western blot was conducted using primary antibodies to evaluate the expression of the various proteins for each condition. Total Cx43 (#3512S; Cell Signaling Technology), and connexin 43 phosphorylated at residue Serine 368 (pCx43s368) (#3511S; Cell Signaling Technology), a less-functional Cx43, were used to probe for cell communication. Troponin T-C (sc-8121; Santa Cruz Biotechnology) and SERCA2a (ab2861; Abcam) were used to probe for cell contractility. Akt (pan) (#4691S; Cell Signaling Technology), Akt1 (#2938S; Cell Signaling Technologies), pAktSer473 (#4058S; Cell Signaling Technologies), and pAktThr308 (#2965; Cell Signaling Technologies) were probed for cardiac growth signaling pathway. Calsequestrin-2 (#C3868; Sigma-Aldrich) and β-actin (#A5331; Sigma-Aldrich) were used to normalize each lane to cardiomyocyte[37] and total cell content, respectively. Calsequestrin-2 expression has been shown previously to not change during physiological or pathological hypertrophy,[37] indicating that it is a good control for cardiomyocytes. Antibodies were used at a 1/1000 dilution for primary antibodies and a 1/5000 dilution for secondary antibodies. The blots were blocked with 5% BSA in tris-buffered saline with Tween 20, and the appropriate horseradish-peroxide-conjugated secondaries (Invitrogen, Inc.) were used to label blots for enhanced chemiluminescence assay. Images were acquired on the G:Box Chemi XR5 (Syngene, Cambridge, United Kingdom). Expression intensities were analyzed using Image J (NIH, Bethesda, MD). Expression intensities for each protein were normalized to the mean expression intensity per blot for that respective protein to allow for comparison between blots.[33]

Statistical analysis

All results are presented as mean ± standard deviation. Differences between conditions were compared using a one-way ANOVA with Tukey's *post hoc* test for all measurements. Statistical significance was determined as $p<0.05$.

Results

Immunohistochemistry

Constructs were stained for troponin I, smooth muscle α-actin, Cx43, cardiac α-actin, and Hoescht, a nuclear stain. Sections of the constructs indicate that the alignment of the cells is along the circumferential axis, as previously shown (Fig. 2, purple arrows). Cx43 expression shows connections between myocytes, though punctuated expression indicates a large number of dead cells embedded into the constructs are not being removed. Similar cardiac α-actin expression was

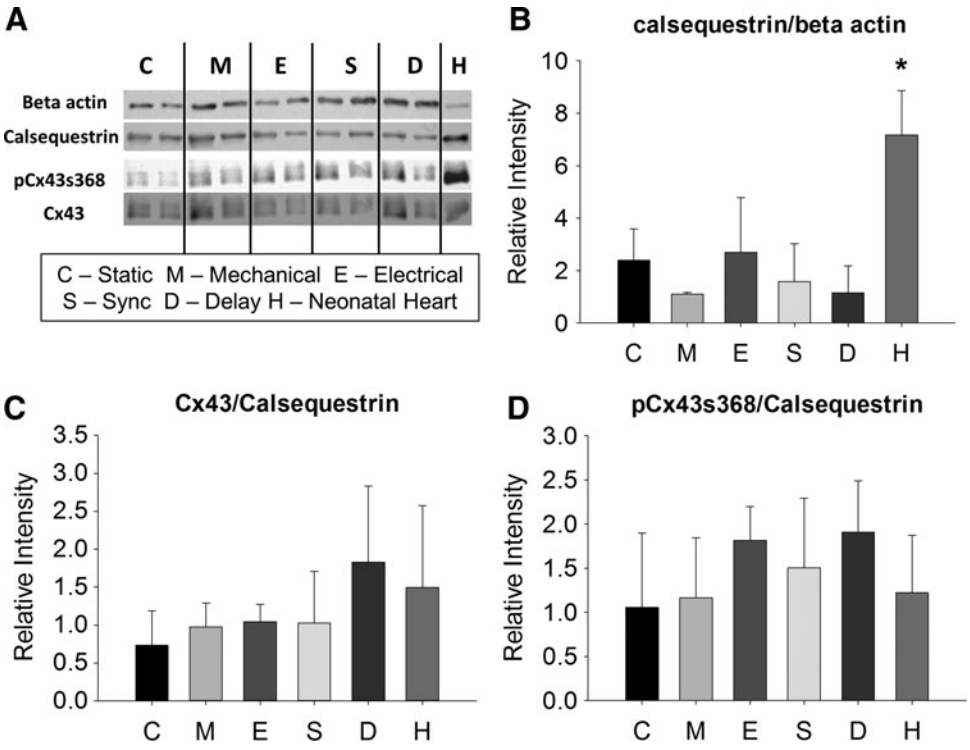

FIG. 6. Cell-to-cell communication protein expression. **(A)** Representative western blots for β-actin, calsequestrin, Cx43, and connexin 43 phosphorylated at residue Serine 368 for all conditions: statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). The control is the neonatal heart lysate (denoted H). All protein expression was normalized to the average expression for that protein per blot to allow for comparisons between blots. Relative intensity of calsequestrin **(B)** normalized to β-actin for all conditions. Relative intensity of Cx43 **(C)** and phosphorylated Cx43 **(D)** normalized to calsequestrin for all conditions. For all figures **(B–D)**, *denotes statistically different than all other conditions ($p<0.05$).

observed between all constructs. This correlates with live/dead flow cytometry data, which indicates that ~84.0% ± 0.8% of the cells in the construct are alive after 2 weeks of culture (data not shown).

Final cell count

DNA quantification of all conditions was used to determine the final number of cells per construct of all conditions after 2 weeks of culture (Fig. 3A). Though 5 million cells were encapsulated into each construct initially, only 3.92 ± 0.87 million cells remained after 2 h ($n=6$) and ~1.75 million cells remained in the static constructs after 2 weeks of culture (30% of initial). A large cell loss was observed in all conditions that is consistent with previous literature.[33] Mechanically stimulated constructs (72.5% ± 20.5%) had a statistically greater percentage of cells as compared with the other conditions (~45.1% ± 9.56%) ($p<0.05$). The percentage of cardiomyocytes after day 14 was similar in all conditions with ~41.2% ± 13.7% of cells being cardiomyocytes (Fig. 3B).

Twitch force and contraction dynamics

All constructs were analyzed for twitch force after 2 weeks of culture in a custom twitch force measuring system during general field stimulation at 0.5 Hz (Fig. 4A). The observed twitch force for statically cultured constructs was 2.08 ± 0.77 mN. All stimulated constructs had a statistically significantly improved contraction force over the statically cultured conditions. The mechanically stimulated constructs exhibited a twitch force of 3.45 ± 0.6 mN, the electrically stimulated constructs exhibited a twitch force of 2.95 ± 1.08 mN, the synchronized condition exhibited a twitch force of 3.18 ± 1.32 mN, and the delayed condition exhibited a twitch force of 3.29 ± 1.09 mN. Figure 3B shows force per cell, which was calculated as the twitch force normalized by the final cell count per construct (Fig. 3). Electrical stimulation improves the force per cell, though not statistically so. No differences were observed in force generation times between the conditions (Fig. 5B). Statically cultured constructs had a statistically smaller force generation rate than all stimulated conditions (Fig. 5C) and a statistically greater force relaxation time than the mechanically stimulated constructs ($p<0.05$) (Fig. 5D), potentially indicating alterations in the excitation–contraction coupling in the static constructs. All stimulated conditions had a statistically greater force relaxation rate than the statically cultured constructs ($p<0.05$) (Fig. 5E).

Expression of cardiac maturation proteins

Previous research from our group has demonstrated that alignment improves Cx43 expression.[33] Representative western blots of β-actin, calsequestrin-2, Cx43, and connexin 43 phosphorylated at residue Serine 368 (pCx43s368), a less-active form of Cx43, are shown for static, mechanically stimulated, electrically stimulated, synchronized, and delayed conditions (Fig. 6A). Figure 6B indicates that all constructs had a statistically less-relative expression of calsequestrin to β-actin as compared with the neonatal heart control. No differences in Cx43 to calsequestrin expression levels (Fig. 6C) or pCx43s368 to calsequestrin levels (Fig. 6D) were observed between conditions.

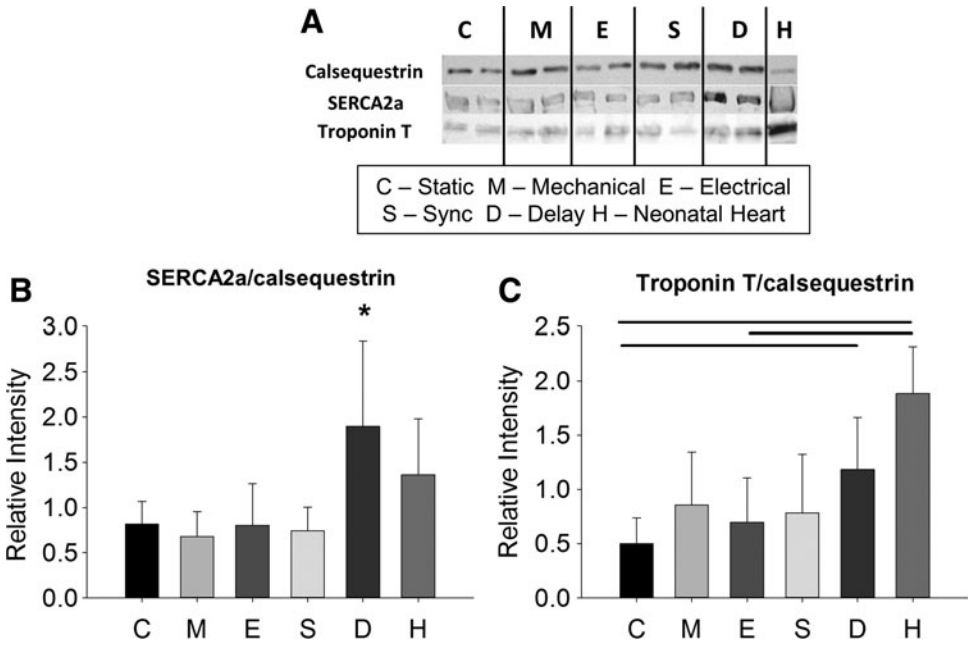

FIG. 7. Contractile and calcium handling proteins. **(A)** Representative western blots for calsequestrin, SERCA2a, and troponin T for all conditions: statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). The control is the neonatal heart lysate (denoted H). All protein expression was normalized to the average expression for that protein per blot to allow for comparisons between blots. Relative intensity of SERCA2a **(B)** and troponin T **(C)** normalized to calsequestrin for all conditions. For figures **(B, C)**, the lines show statistical significance of $p<0.05$ and *denotes statistically different than all other conditions ($p<0.05$).

SERCA2a, a calcium handling protein, pumps intracellular calcium back into the sarcoplasmic reticulum, which is required for muscle relaxation, and replenishes the calcium required for the next contraction.[38] Troponin T is a part of the complex that regulates cardiac muscle contraction and myofibril formation.[39] Representative blots of calsequestrin, SERCA, and troponin T are shown in Figure 7A. The delayed conditioned constructs had a statistically greater expression of SERCA2a relative to calsequestrin than the other constructs as well as the neonatal heart control ($p < 0.05$) (Fig. 7B). Further, the relative troponin T to calsequestrin levels were greater in the delayed condition and the neonatal heart than in the static condition ($p < 0.05$) (Fig. 7C), but not for other groups.

Expression of hypertrophic pathway proteins

Akt is an important target in the IGF1-PI3K growth signaling pathway[40] and is often used as an indicator of physiological hypertrophy.[41] Representative blots of β-actin, calsequestrin, Akt (pan), Akt1, and activated phosphorylated Akt (pAktSer473 and pAktThr308) are shown in Figure 8A. The delayed condition had a statistically greater Akt (pan) to calsequestrin expression as compared with the electrical condition ($p < 0.05$) while the neonatal heart control had statistically less Akt (pan) expression as compared with all construct groups ($p < 0.05$) (Fig. 8B). We further probed for Akt1, which is the specific Akt isoform related to cardiac hypertrophy.[42] The delayed condition had statistically greater Akt1 to calsequestrin expression than the static, mechanically stimulated, and electrically stimulated culture conditions ($p < 0.05$) (Fig. 8C). The phosphorylated Akt at the Ser473 residue (pAktSer473) and phosphorylated Akt at the Thr308 residue (pAktThr308) were probed to determine the activated Akt expression (Fig. 8D, E). No differences in pAktSer473 between conditions were observed, though a trend of increased pAktSer473 was observed in the delayed condition over the static condition, though not quite statistically significant ($p < 0.1$). An increase in the pAktThr308 expression was observed in the mechanical and delayed conditions over the neonatal heart control ($p < 0.05$) (Fig. 8B), but not for other groups.

Discussion

The physical stimulation of engineered cardiac tissue has generated significant interest in recent years as a method of developing constructs that more closely mimic healthy

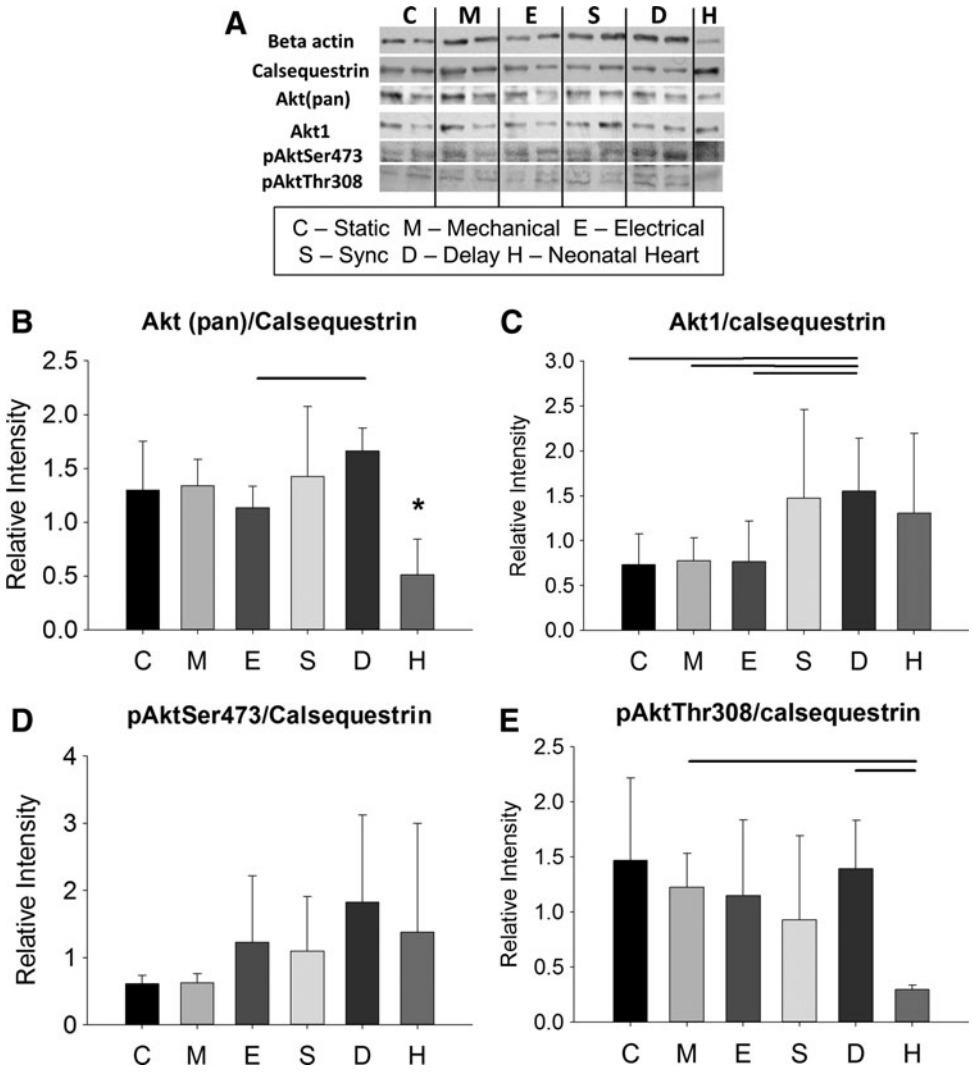

FIG. 8. Growth signaling proteins. **(A)** Representative western blots for β-actin, calsequestrin, Akt (pan), Akt1, pAktSer473, and pAktThr308 for all conditions: statically cultured (denoted C), mechanically stretched (denoted M), electrically paced (denoted E), synchronized combined electromechanical stimulation (denoted S), and delayed combined electromechanical stimulation (denoted D). The control is the neonatal heart lysate (denoted H). All protein expression was normalized to the average expression for that protein per blot to allow for comparisons between blots. Relative intensity of Akt (pan) **(B)**, Akt1 **(C)**, pAktSer473 **(D)**, and pAktThr308 **(E)** normalized to calsequestrin for all conditions. For all figures **(B–E)**, the lines show statistical significance of $p < 0.05$ and *denotes statistically different than all other conditions ($p < 0.05$).

myocardium.[6] Though mechanical stimulation[43] and electrical stimulation[16] have been used as standard methods of stimulation, and previous bioreactors have been developed that incorporate both stimuli,[14,25,26] to date, no one has carried out a comparison between the effects of mechanical stimulation and electrical stimulation in the same system. Likewise, no study has investigated combined stimulation of cardiac constructs that mimics the *in vivo* environment and could lead to engineered tissue that generates the level of force present in healthy hearts. To study the effects of combined stimulation, we have created a novel bioreactor system with the ability to precisely control the timing of mechanical and electrical stimulation of the constructs. We hypothesized that delayed electromechanical stimulation that mimicked the isovolumic contraction would best improve the function of our engineered tissue. Our results indicate that electrical and mechanical stimulation alone improved twitch force similarly over static culture with mechanical stimulation having a statistically greater final cell count in the tissue, but no other significant differences between the two were noted. Further, although all stimulated conditions had statistically significantly improved twitch force over statically cultured constructs ($p < 0.05$), the mechanical-only stimulation had the highest forces, though not statistically significant over other groups. In addition, the delayed combined stimulation improved SERCA2a, troponin T, and Akt protein expression that are all important for cellular contractility and hypertrophic growth. These findings are discussed in more detail in the following paragraphs.

In the present study, the culture of the constructs over the 2-week period resulted in a low cell viability/retention such that only $\sim$45% of the cells initially entrapped in the gels remained, similar to previous findings.[33] The cell loss is likely caused by the isolation and encapsulation process itself. Fibrin has been shown previously to contain RGD and AGDV sites to interact with integrins,[44] allowing for cell adhesion. However, upon initial casting, the constructs are very hydrated and porous due to the low 3.3 mg/mL fibrin concentration and it is likely that some cells could have been lost out of the construct upon its removal from the outer mold casing. Indeed, previous unpublished research by our lab has shown that $\sim$80% of the cells encapsulated into the construct are maintained in the initial construct creation process. After 2 weeks of culture, $\sim 45.0\% \pm 9.6\%$ of the cells encapsulated were maintained with $\sim 84.0\% \pm 0.8\%$ of the cells alive, as demonstrated by flow (data not shown) (Fig. 3B). This result mirrors previous reports, which showed an overall decrease in cell number in constructs, with an increase in the ratio of fibroblasts to cardiomyocytes over time in culture.[45] Our histological analysis results indicate that after day 14, $\sim 41.2\% \pm 13.7\%$ of the cells in the constructs were cardiomyocytes, similar to previous findings.[33] Indeed, our histological sections indicate that all conditions have similar patterns of all cell types and numbers.

Mechanically stimulated constructs had the greatest twitch force (Fig. 4A), which is likely due to the significantly higher final cell number, and thus, higher myocyte numbers, as compared with the other conditions ($p < 0.01$) (Fig. 3A). Though mechanical stimulation has been known to have a negative effect on the proliferation rates of fibroblasts at 5 Hz,[46] it has been shown to have a positive effect when held at a constant tension.[47] It is possible that mechanical stimulation helps maintain cocultured cells in culture better by improving the transport of nutrients through the fibrin scaffold, although we would expect that all groups with mechanical stimulation (including the combined stimulation groups) would see this effect, which is not the case. Though short-term electrical stimulation has also been previously shown to induce cardiac fibroblast proliferation,[48] the extended regimen of electrically pacing constructs for 2 weeks and/or the level of electrical field generated in this study could have had a negative effect on cell numbers.

Previous studies have shown that cyclic mechanical stretch improves cardiomyocyte orientation[49] and proliferation,[50] and promotes hypertrophy.[51] Further, active forces between 0.05 and 2 mN have been previously reported after mechanical stimulation[52]; Cx43 mRNA expression is increased threefold[49,53] and phosphorylated Akt expression increased 40% in mechanically stretched constructs when compared with static conditions.[50] Electrical stimulation has also been shown to improve the conductive and contractile properties of constructs.[16] Radisic *et al.* showed that contraction amplitudes, as measured by fractional area change of the construct, were sevenfold higher in electrically stimulated constructs than in nonstimulated constructs.[16] Indeed, our results indicate that biphasic electrical stimulation increased Cx43 and troponin T expression levels when compared with the static conditions.[54] While the degree to which our constructs functionally improved following physical stimulation (38% increase in twitch force) is less than in previously published literature, the magnitude of all of our forces, including the static condition, is significantly higher than what has been reported in those studies. Our results indicate that electrical and mechanical stimulation alone resulted in similar twitch forces, though they affect the construct development in different manners. Because mechanically stimulated constructs had higher cell numbers, this led to a low force per cell. There was a trend for the electrically stimulated constructs to have a higher force per cell than the mechanically stimulated constructs, but this was not statistically significant ($p < 0.06$). Further, the expression of cell–cell coupling and contractile proteins was not significantly different between the two types of stimuli. Based on previous studies, increases in the Cx43 and Akt expression would be expected for both types of stimulation. Though the protein expression for both mechanical and electrical stimulation tended to increase as compared with the static controls, no statistical differences were observed between electrical and mechanical conditions. It is important to note that although no differences were present in the functional measurements that were made in this study, it is possible that differences might exist in other functional measurements, such as excitation threshold or maximum capture rate. Further, because the conditions used in this study were based on values taken from literature to enable direct comparisons to previous literature, there may be differences in the specifics of our system that warrant future optimization.

Improved contractility in engineered constructs is caused by a combination of many different factors, including myocyte number, myocyte hypertrophy, and increased cell–cell signaling. In our study, all groups had similar levels of calsequestrin to β-actin and histological sections indicated

similar percentages of cardiomyocytes between groups, indicating that stimulation had no effect on the myocyte concentration in the construct over the 2-week culture period. Because only the outer cardiomyocytes are likely to sense the general field stimulation used to stimulate the constructs in our bioreactor and during our force measurements, functional gap junctions are required to propagate the signal throughout the thickness of the construct.[55] Delayed constructs had a higher relative Cx43 to calsequestrin expression level as compared with the static condition, but this did not quite achieve statistical significance ($p < 0.09$). pCx43Ser368 is known to be a less-functional form of Cx43, as demonstrated by reduced conductivity,[56] as this phosphorylation event marks the gap junction for internalization and breakdown by the ubiquitin proteosome system.[57] While previous work by our lab had demonstrated differences in the level of pCx43Ser368 that were correlated with alterations in contractile force as a function of construct alignment,[33] in the current study, no differences were observed between the pCx43Ser368 to β-actin ratio or the ratio between inactivated pCx43Ser368 to total Cx43 (data not shown), indicating that changes in the overall expression/function of the gap junctions did not account for the differences in contractile function observed between conditions.

To ascertain the potential cause of the increased contractile forces, we investigated several other proteins important in contraction. Cardiac sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) is the protein responsible for pumping calcium back into the sarcoplasmic reticulum during diastole.[38] It is important for the excitation–contraction coupling in the heart, and its expression increases through development[58] and decreases in heart failure,[59] resulting in impaired contractility and fatal arrhythmias.[60] Increased expression was shown to enhance left ventricular mechanics and improve contractile efficiency[61] and electrical stability after MI.[62] Previous studies have shown that isotonic contraction improves SERCA2a expression over isometric contraction.[63] Therefore, it is likely that the increase in SERCA2a expression in the delayed conditioned constructs as compared with all other constructs (as well as the neonatal heart control) ($p < 0.05$) was a direct result of the switch between isometric and isotonic contraction, which had a positive effect on the maturation and contractility of the engineered tissue by improving the excitation–contraction coupling. This improvement in calcium handling also likely resulted in the increase in force generation and relaxation rates as compared with the static condition (Fig. 5C, E) ($p < 0.05$). However, when electromechanical stimulation occurs simultaneously, the constructs respond similarly to mechanical stimulation alone. This response is likely due to the fact that synchronized condition induces only isometric contraction, which mirrors that of mechanical stimulation alone, and thus explains why no increase in SERCA2a expression was observed in the synchronized condition as compared with the statically cultured condition.

Troponin T (cTnT), a thin-filament regulatory protein that is responsible for sarcomere assembly[64] and affects calcium sensitivity,[39] which is necessary for the generation of coordinated force in cardiac tissue,[65] was also explored as a potential cause of the increased contractile forces. Troponin T is associated with increased calcium sensitivity[66] and has been shown to increase with development.[67,68] Previous studies have shown that a loss of cTnT prevents the assembly of sarcomeres and renders the heart muscle nonfunctional[64] and mutations are associated with dilated cardiomyopathy.[69] The delayed condition had an increase in relative cTnT to calsequestrin expression as compared with the statically cultured constructs ($p < 0.05$). The increase in troponin T and SERCA2a indicates that the delayed condition had increased calcium sensitivity and improved calcium handling, which is necessary for maturation. No differences were observed in the synchronized combined electromechanical–stimulated conditions as compared with the other conditions.

Akt, a well-characterized protein in the IGF1-PI3K growth signaling pathway, has been shown to be required for physiological cardiac development.[40,41] It promotes the survival of cardiomyocytes *in vitro* and protects the myocardium after MI.[70] The cardiac-specific Akt1 isoform improves calcium handling,[71] enhances contractile function and organization of cardiac structures,[71] and has been shown previously to upregulate SERCA2a expression.[72] Because of the increase in SERCA2a expression observed in the delayed condition, we probed Akt levels to determine the influence of hypertrophy on the differences in contractile function. In our study, the delayed condition constructs had greater relative expression of Akt (pan) to calsequestrin than the electrical condition ($p < 0.05$), and a trend of increased expression over the mechanical condition ($p < 0.09$). Further, the delayed condition had a greater expression of Akt1 to calsequestrin as compared with the statically cultured and singular stimulation constructs ($p < 0.05$). This increase in Akt1 and cTnT expression indicates that mimicking physical environment during the isovolumic contraction with the delayed condition could help induce maturation via physiological hypertrophy. An increase in the Akt1 expression in the synchronized electromechanically stimulated conditions was also observed, but this increase was not statistically significant. Activation of Akt occurs with phosphorylation of the protein and, in this study, we probed two of these phosphorylation sites: Ser473 (pAktSer473) and Thr308 (pAktThr308).[73,74] Increased levels of pAktThr308 were observed in the delayed and mechanical-only conditions as compared with the neonatal control ($p < 0.05$) and while no statistical difference was observed between conditions for pAktSer473 expression, a trend of increased expression with high variability was observed with conditions containing electrical stimulation. Activation of Akt as measured by both Ser473 and Thr308 indicates that the physiological hypertrophic pathway is a likely cause of the improvements in contractile function observed in the delayed combined stimulated conditions, although future experimentation is needed to confirm this finding. Future experiments will explore other hypertrophic pathway proteins to better clarify the role of hypertrophy in the observed effects of combined stimulation.

There are a few limitations of our study. While the twitch forces observed over 2 weeks in the delayed condition were not significantly different than the other stimulated conditions, it is possible that the differences in twitch force will be more distinct over a longer duration study. In addition to looking at longer culture periods, the hypertrophic signaling pathways should be further explored to better characterize how the change from isometric to isotonic contraction

affects the development of the tissue construct and which pathways are playing the greatest role. It is also important to note that the parameters for the stimulation used in this study were drawn from literature values and there was no attempt to assess any effect of changing the parameters (e.g., voltage, % strain, and duty cycle). Further studies are needed to determine whether optimization of these parameters could further distinguish the differences between the stimulation types.

In summary, we compared the effects of electrical and mechanical stimulation alone as well as studied the effects of the timing of combined electromechanical stimulation on engineered cardiac tissue. Though electrical and mechanical stimulation are established methods of enhancing function of engineered cardiac tissue, no study had previously compared the two stimulation methods. We demonstrated that no functional differences were observed between mechanical and electrical stimulation alone, indicating that they affect the construct similarly in our system. Further, delayed combined electromechanical stimulation, which mimics the physical environment observed during the isovolumic contraction, improves SERCA2a and TnT, cellular proteins responsible for calcium handling and contractility while the synchronized condition did not improve them, indicating that timing between electrical and mechanical stimulation is an important factor that is critical to its effect on engineered cardiac construct function.

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Disclosure Statement

No competing financial interests exist.

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