# Self-organization of rat cardiac cells into contractile 3-D cardiac tissue

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## ABSTRACT

The mammalian heart is not known to regenerate following injury. Therefore, there is great interest in developing viable tissue-based models for cardiac assist. Recent years have brought numerous advances in the development of scaffold-based models of cardiac tissue, but a self-organizing model has yet to be described. Here, we report the development of an in vitro cardiac tissue without scaffolding materials in the contractile region. Using an optimal concentration of the adhesion molecule laminin, a confluent layer of neonatal rat cardiomyogenic cells can be induced to self-organize into a cylindrical construct, resembling a papillary muscle, which we have termed a *cardioid*. Like endogenous heart tissue, cardioids contract spontaneously and can be electrically paced between 1 and 5 Hz indefinitely without fatigue. These engineered cardiac tissues also show an increased rate of spontaneous contraction (chronotropy), increased rate of relaxation (lusitropy), and increased force production (inotropy) in response to epinephrine. Cardioids have a developmental protein phenotype that expresses both α- and β-tropomyosin, very low levels of SERCA2a, and very little of the mature isoform of cardiac troponin T.

Key words: tissue engineering • heart development

The long-term goal of cardiac tissue engineering is to develop tissue equivalents to augment or replace failing hearts. Currently, ~90% of Americans who could benefit from a heart transplant do not have the opportunity (1) due to the extremely limited availability of suitable donor organs (2). Therefore, the incentive to develop alternative sources of cardiac tissue is very strong. Furthermore, ex vivo cardiac tissue models will serve as important tools for testing questions in developmental biology and determining the functional effects of pharmacological agents that may have deleterious effects on the cardiovascular system.

Although scaffold-based cardiac tissue constructs have been reported (3–14), the creation of a functional self-organizing cardiac muscle construct has proven elusive. The many reasons for this include the fact that cardiac myocytes are post-mitotic (they no longer undergo cell division to produce greater numbers of cells). Therefore, it is difficult or impossible to amplify cardiomyocytes in culture. Recently, skeletal muscle has been engineered into self-organizing 3-D tissue constructs (15–17). These contractile constructs do not use an artificial scaffold in their contractile region; rather, the tissue self-organizes from a cohesive monolayer of myotubes and fibroblasts. The result is a 25-fold increase in force production over scaffold-based muscle systems when normalized by cross-sectional area. A similar improvement of cardiac function would be an important step in the development of functional cardiac tissue in vitro.

The ability to create cardiac tissue without the mechanical restriction of scaffolds has many important scientific applications. Even small functional cardiac tissue constructs could be used to study the effects of gene therapy or various drugs on cardiac tissue formation and contractility. On a larger scale, the greater specific force of scaffold-free engineered cardiac muscle could be used to improve the existing techniques for the development of viable replacement tissue for the treatment of heart failure (1). This realization has recently led to the creation of cardiac sheets (18, 19), electrically coupled layers of neonatal rat cardiomyocytes stacked on top of each other using a temperature-sensitive polymer, and myocardial spheroids, electrically coupled beating heart cultures (20). Although these techniques represent a significant innovation, their functional capacity has yet to be described, therefore it is difficult to functionally compare them with previous forms of engineered cardiac tissue.

Here, we describe a method for culturing cardiomyocytes and fibroblasts in such a way as to promote the self-organization of a contractile cardiac muscle organ in culture. The cardiomyocytes form a cylindrical construct that is spontaneously contractile; can be electrically induced to contract and show positive inotropy, chronotropy, and lusitropy with epinephrine treatment; and express developmental isoforms of many cardiac-specific proteins. We designate these tissue constructs *cardioids*, as they are similar to embryonic mammalian cardiac muscle.

## MATERIALS AND METHODS

### Materials

Reagents for SDS-PAGE were from Cambrex Bioscience (Rokland, ME). The bicinchoninic (BCA) protein assay kit, horseradish peroxidase-conjugated secondary antibodies, and WestDura chemiluminescent reagents were purchased from Pierce (Rockford, IL). Goat polyclonal antibodies directed against cardiac troponin T (cTnT) and SERCA2a were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody directed against slow myosin heavy chain was from Novocastra (Newcastle upon Tyne, UK), and the mouse tropomyosin antibody was a gift from Joseph Metzger (University of Michigan). All other reagents were purchased from Sigma Chemical (St. Louis, MO).

### Preparation of cardioids

The development of self-organizing cardiac tissues was based on a previously reported method for skeletal muscle engineering (16, 17). Briefly, culture plates (35 mm diameter) were coated

with 1.5 ml of SYLGARD (polydimethylsiloxane, PDMS) and allowed to cure for 2 weeks. The adhesion and migration of cells on the hydrophobic PDMS substrate material were promoted using the extracellular matrix protein laminin. Seven days prior to plating cells, the PDMS-coated plates were layered with 3 ml of phosphate-buffered saline (PBS) containing 6 µg of laminin. The PBS was evaporated overnight in a biological safety cabinet, and the residual salt was washed off the next day leaving a layer of laminin coating the PDMS. Two anchors, made from 6-mm-long, laminin-coated braided silk suture, were then pinned into place at either end of the plate (12 mm apart), and the plates were filled with 1 ml of plating media (67% DMEM, 17% M199, 5% FBS, 10% horse serum, and 1% antibiotic/antimycotic) and placed in the incubator until the cells were ready.

Cells were dissociated from 2–3 day old neonatal rat ventricles using 80 U/ml collagenase type II and 0.6 mg/ml pancreatin, dissolved in 25 ml 1× ADS (11.6 mM NaCl, 2.0 mM HEPES, 0.1 mM $Na_2PO_4$, 0.55 mM glucose, 0.54 mM KCl, and 0.08 mM $MgSO_4$ pH=7.2). The isolated cells from the hearts were counted using a hemocytometer, plated at high density (0.4 million cells/cm$^2$) and allowed to adhere to the plate for 2 days prior to the first media change. Thereafter, the culture media was changed every other day for the extent of the experiment. After 48 h, the cells began to spontaneously contract. By 7 days, the fibroblast cells had proliferated to form a confluent layer that contracted as a syncytium and began to detach from the PDMS substrate, starting at the periphery of the plate (Fig. 1A). Delamination of the monolayer progressed and generally accelerated after each subsequent feeding (Fig. 1B). Ten days after plating, the cells completely detached from the PDMS substrate but remained attached to the two anchors, forming a cylindrical 3-D self-organized tissue construct (Fig. 1C). Cardioids produced using the methodology described above have been generated from 25–30 independent litters.

**Electrical stimulation of cardioids and force determination**

The contractility of the tissue constructs was evaluated as described earlier (17). During the 15 min measurement of contractile properties, the temperature of the engineered muscles was maintained at 37 ± 1°C using a heated aluminum platform. One of the anchors was unpinned from the substrate and affixed to a force transducer using canning wax. The length of the specimen was adjusted to restore the original specimen length while in culture. Wire electrodes were placed parallel to the long axis of the specimen on both sides, and electrical pulses were applied to the specimen via the parallel wire electrodes (Fig. 1D). The optical force transducer is one of our own design with a force resolution of between 1.4 µ*N* and 2.0 m*N* (21). The force transducer position was adjustable on three axes by use of a micromanipulator (Newport MT-XYZ; Newport, Irvine, CA). The stimulation pulses were square and bipolar. Stimulation was controlled using a LabVIEW data acquisition system (National Instruments, Austin, TX) and amplified using a Crown DC-300A amplifier. The system permitted control of pulse width, pulse amplitude (voltage), pulse frequency, and duration of each pulse train. Cardioids were stimulated at a pulse amplitude of 10 V with varying pulse frequencies from 1 to 10 Hz. To determine the effects of calcium and epinephrine (dissolved in calcium-free media containing 1 mM EGTA) on cardioid contractility, the constructs were stimulated at 1 Hz in media containing from 0 to 0.01 M calcium and 0.2 to 1.2 µg/ml epinephrine.

## Electron microscopy

After electrical testing, the cardioids were fixed for 4 h at 4°C in a 0.1 M sodium cacodylate buffer solution pH = 7.4 (Electron Microscopy Sciences, Fort Washington, PA) containing 3% formaldehyde/glutaraldehyde. After fixation, the samples were rinsed with cacodylate buffer (pH=7.4) containing 7.5% sucrose, postfixed in 1% osmium tetroxide, dehydrated, and embedded in EPON (Ted Pella Inc., Redding, CA, Eponate 12 resin), and sectioned into 50 nm longitudinal strips.

## Western blots

Embryonic heart (15 days post-coital, dpc), neonatal heart, cardioid, adult heart, slow skeletal (soleus), and fast skeletal (extensor digitorum longus, EDL) muscles were homogenized in 9 volumes of ice-cold buffer containing 10 mM $MgCl_2$, 10 mM $KH_2PO_4$, 1 mM EDTA, 5 mM EGTA, and freshly added 50 mM β-glycerophosphate, 2.5 mM PMSF, 10 μg/ml leupeptin hemisulfate, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate. Aliquots of the homogenate were solubolized in Laemmli sample buffer, subjected to SDS-PAGE, and electrophoretically transferred to nitrocellulose in a genie electrophoretic transfer apparatus (Idea Scientific). Membranes were blocked overnight at 4°C with 5% non-fat dry milk in tris-buffered saline containing 0.1% Tween-20. The blots were probed with primary antibodies for 1 h at room temperature followed by incubation with the appropriate horseradish peroxidase conjugated anti-IgG antibody. Antibody-bound protein was detected using West Dura chemiluminesence and a BioRad chemidoc system.

## RESULTS

### Monolayer culture, delamination, and self-organization of cardioids

Within one week of plating, a monolayer of cardiac cells, including cardiomyocytes and cardiac fibroblasts, had developed and appeared to contract as a syncytium. Approximately 180 h after plating, the monolayer of cells began to detach from the periphery of the substrate. Once the cell monolayer began to detach from the substrate, the delamination process was typically quite progressive. With each active contraction, the boundary of delamination moved radially inward toward the center of the plate. This, in turn, resulted in additional mechanical strain on the cells that were still attached to the substrate in proximity to the delamination boundary, further accelerating the delamination process. The peak rate of delamination occurred at the point at which the delamination front had progressed ~50% of the radial distance from the outer circumference of the culture substrate. In several cases, the monolayer delaminated as much as 50 μm during each active contraction. At the peak rate, the process of delamination and self-organization of the tissue monolayer into a cylindrical 3-D structure was visible to the naked eye. With each active contraction, the entire detached portion of the monolayer structure undulated, progressively releasing the remaining cells from the substrate below while remaining attached to the lamin-coated suture anchors at each end. The delamination of several of the plates was captured on digital video and can be viewed at www.umich.edu/~bobden/cardiac_tissue_engineering.html. The process of cell monolayer delamination required approximately five days to run to completion, so intermediate stages of

monolayer delamination were readily observed (Fig. 1). The resulting tissue was 24 mm long and ~100 μm in diameter. These constructs were stable in this 3-D form for up to 60 days in culture.

## Ultrastructure of cardioids

Light micrographs of the cardioids stained with hematoxylin and eosin show that the cardioids were composed of a densely packed collection of cells (Fig. 2). At higher magnification, connected cell groups were clearly visible and were separated by acellular material and extracellular matrix. Sections stained with the MF20 antibody that recognizes all isoforms of type II myosin heavy chain and counterstained with DAPI to show all nuclei, showed that greater than 70% of the cells expressed the myosin heavy chain and were likely cardiomyocytes (Fig. 2C, D).

As in the light micrographs, cross-sectional electron micrographs of the cardioids (Fig. 3) showed groups of cells all demonstrating the hexagonal array of contractile proteins. In longitudinal sections, striations characteristic of skeletal and cardiac muscle were clearly evident, demonstrating the organization of the contractile proteins. As in control heart tissue, areas rich in mitochondria flanked the regions of contractile machinery. Each cell had a single prominent nucleus and was surrounded by regions of connective tissue. Between the cardiomyocytes in series and in parallel were electron-dense regions, indicating that adherens and gap junctions, respectively, had formed between the cardiac myocytes.

## Contractile behavior of cardioids

Within 48 h of complete delamination from the PDMS substrate, cardioids were removed from the incubator for quantitative assessment of contractility. The newly formed tissues exhibited spontaneous contractility and were electrically excitable between parallel platinum wire electrodes (Fig. 4). The cardioids could be entrained to contract with a "pacemaker" set to 1–2 Hz (Fig. 4B), for an indefinite period without detectable fatigue. By comparison, similar skeletal muscle constructs (myooids) require 30 to 120 s between peak twitches to once again be able to produce an equivalent peak twitch force (17). The peak active force was 141.1±34.06 μN, with an average baseline force of ~260 μN. Normalizing by the total cross-sectional area of the smallest diameter of each construct, the resulting average specific force (stress) generation was $66.2 \pm 29.81$ kN/m$^2$. The resting force is high relative to the active force produced by the cardioids. This is likely due to the presence of significant numbers of fibroblasts in the constructs and the ECM they generate (15).

At any given calcium concentration, both spontaneous and electrically induced contractions produced the same amount of absolute force. However, varying the calcium concentration modulated peak contractile force (Fig. 5). The ability of cardioids to increase force production in response to increasing calcium suggests that they behave like heart muscle in their dependence upon extracellular calcium for contraction.

To test the contractile response to cardioactive agents, cardioids were stimulated in the presence of epinephrine. As expected, epinephrine treatment increased the rate of spontaneous contraction in a concentration-dependent manner (Fig. 6). Unexpectedly, in serum- and calcium-containing media, epinephrine had no effect on either the spontaneous or electrically elicited force produced

by the cardioids. However, when cardioids were electrically stimulated at 1 Hz in a serum-free media containing a half-maximal concentration of calcium, epinephrine induced a dose-dependant response for both inotropy and lusitropy (Fig. 7). The force produced by the cardioids treated with maximal epinephrine increased 253 ± 58%, while the half-relaxation time decreased 32 ± 14%.

During the epinephrine experiments, we observed that the cardioids occasionally produced spontaneous contractions that did not respond to low-level external electrical stimulation (Fig. 8). The force produced by this type of spontaneous contraction was lower than normal and was characterized by less well-synchronized contractions of the myocytes. This behavior is strikingly similar to fibrillation in cardiac muscle. As occurs in vivo, the cardioids could only be returned to normal contractile function by stimulating either with a greater voltage or when the cardioid was refractory (force was ~1/4 of maximal).

**Phenotype of the cardioids**

To determine the developmental state of the cardioids, Western blots were performed to study the isoforms of cardiac troponin T (cTnT) and tropomyosin, and the levels of SERCA2a. Cardioids express both α- and β-tropomyosin (Fig. 9). We observed three specific bands for cTnT in cardiac tissue. The fastest migrating band (at ~23 KDa) was present only in the cardioids, embryonic day 15 (E15) hearts, and neonatal heart. In contrast, the intensity of the ~40 KDa band was highest in the mature heart, less neonatal and absent from the E15 hearts and cardioids. The levels of SERCA2a are similarly highest in the adult heart with progressively less in the neonatal, embryonic, and cardioid tissue.

To determine how the protein phenotype compared with 2-D cardiac cell cultures, purified cardiomyocytes were collected 4 days after plating and the level of cTnT, tropomyosin, SERCA2a, and slow/cardiac myosin heavy chain was determined. At 4 days, 2-D cardiomyocytes already begin to express high levels of β-tropomyosin (Fig. 10). Also at this time, the ~23 KDa cTnT appears. Both the cardioids and the 2-D cardiomyocytes have high levels of cardiac MHC but the 4-day-old cells have significantly higher SERCA2.

DISCUSSION

A great deal of interest has focused on the development of functional cardiac tissue equivalents. The lack of sufficient donor tissue means that many patients who would benefit from cardiac transplant lack such an opportunity. The initial emphasis in this area of research was on scaffold-based processes, in which cardiomyocytes were seeded in a scaffold material and implanted into an animal and the outcome was determined (22). Although these implants have been successful, cell-free controls have not been performed to see whether the improved function was the result of the action of the cells or simply due to the presence of the matrix. Furthermore, the biology of the implanted cells has not been described. Another concern is the low force per cross-sectional area of cardiac grafts. To address this concern, Shimizu et al. (18) have described a technique to produce layers of electrically and mechanically coupled cardiomyocytes. Sheets up to three-monolayers-thick can be formed by using this method and the broad, flat tissue is ideal for implantation. However, it is difficult to functionally evaluate the tissue to determine whether the specific force will be sufficient to function as a cardiac assist.

In this report, we describe conditions that promote the formation of self-organizing contractile cardiac tissues from disaggregated neonatal rat cardiomyocytes. These cardioids self-assemble into papillary-type structures that attach to artificial tendons, spontaneously contract, contract in response to electrical stimulation, generate force, and respond to β-adrenergic stimulation. Cardioids display an early embryonic phenotype, expressing developmental isoforms of several proteins.

The cardiac tissue described here is similar to the engineered heart tissue (EHT) generated by Eschenhagen with a few important differences. EHT are made through a gelation process by mixing cardiomyocytes with a solution containing collagen I and matrigel and then pouring the cells into molds of various sizes and shapes (6, 13, 14, 24, 25). When rectangular molds are used, a piece of silicone tubing covered with Velcro® (the "loop" portion of this commercially available hook-and-loop fastener) is placed at each end of the mold to promote adhesion with the collagen gel. After 7 days in culture, the constructs are placed in a bioreactor that applies a unidirectional stretch (10% stretch at a frequency of 2 Hz). The mechanical stretch promotes the alignment of the cardiomyocytes within the gel and increases the size of the cells as well (24). The resulting 3-D constructs are larger (~29 mg and 830 μm in diameter) and produce more absolute force (750 μN at maximal calcium levels) than the cardioids described here. The larger size of the EHTs is possible because, within the collagen gel, bundles of longitudinally oriented cardiac myocytes range in size from 30 to 100 μm in diameter and are separated by a loose collagen array (14). The areas of collagen between the myofibrillar bundles are thought to serve as channels that allow nutrients and oxygen to permeate the inside of the construct preventing the core from becoming necrotic. Because our cardioid tissues self-organize in the presence of fibroblasts, the cellular density in the cardioids is greater and results in a diffusion distance limitation to the size of the cardioid to a "viable radius" of ~40 μm. Constructs of larger radius invariably have a necrotic core. Although the acellular space allows the EHT to maintain a larger size and therefore greater amounts of absolute force, the normalized force of the EHTs is relatively small, in the range of 2.1–4.3 $kN/m^2$, compared with the $66.2 \pm 29.81$ $kN/m^2$ generated by the cardioids described here. This ~20-fold increase in specific force is a significant improvement on the existing model of cardiac muscle engineering. The power density of contractile tissue (W/kg) is directly related to the specific force generation of the constituent muscle, and high-power densities are essential for adequate cardiac muscle function.

In adult heart tissue, epinephrine increases the force of contraction (positive inotropy), the rate of contraction (positive chronotropy), and the rate of relaxation (positive lusitropy). This behavior has also been described in engineered cardiac muscle (6, 13, 14, 24, 25). However, in normal calcium- and serum-containing media we did not observe either positive inotropy or lusitropy in response to epinephrine. Cardioids do show both positive inotropy and lusitropy when electrically stimulated at 1 Hz in a serum-free media containing half-maximal calcium level. However, the force produced in the presence of epinephrine never exceeded the maximal force produced in basal media. The explanation for this discrepancy between the cardioids and the EHT may lie in the stimulation media and/or the conditioning performed by Zimmermann and his colleagues (13). We have studied the role of the media in this response by stimulating the cardioids in Krebs-Henseleit buffer. Simply changing the buffer resulted in a small but significant inotropic response (data not shown). Stretch of cardiac myocytes is an important growth stimulus (26) and may be required for the maturation of cardiac tissue. This maturation

may include improved response to adrenergic agonists. This hypothesis is supported by the fact that other tissue-engineered cardiac muscle models that have not been stretched show a much smaller increase in force production with epinephrine treatment (7, 10).

One of the most interesting observations was that the cardioids showed classic signs of fibrillation. Fibrillation can only occur at the tissue level since it arises from multiple action potential wavelets reentering electrically coupled cells. This would not be possible in cardioids if they were a collection of independent cells. The most likely explanation for this phenomenon is that the media and the structure of the cardioids provide an electrical path that is conducive to reentry. In low-magnification cross-sectional light- and electron microscopic images (not shown), cardioids appear structurally similar to the previously reported skeletal muscle myooids (17), arranged as a parallel bundle of contractile muscle fibers surrounded by an outer layer of fibroblast cells. We hypothesize that the fibroblast layer on the outside of the cardioids provides sufficient electrical insulation to create a torroid-shaped electrical reentry pathway, through the media on the outside of the cardioid. In fact, when we electrically stimulate our engineered skeletal muscle constructs, there is always a decreasing wave of contractions following the initial stimulus as the pulse is conducted back to the tissue. In the cardioids it appears as if this phenomenon is sometimes sufficient to reactivate the tissue. The fact that this reactivation occurs in a disorganized fashion indicates that the cardiomyocytes within this functional tissue are at different points in the refractory cycle much the same as occurs in vivo.

Although many of the contractile features of heart muscle are observed in cardioids, they are not equivalent to adult cardiac muscle or 4-day-isolated cardiomyocytes. Unlike the adult heart, the cardioids contain both α- and β-tropomyosin, no adult cTnT, and very little SERCA2a. Although none of the hearts collected from animals at any age, including embryonic day 15, express β-tropomyosin, Muthuchamy et al. (27) have previously shown that at the earliest developmental state of cardiac tissue both α- and β-tropomyosin are expressed consistent with what we observe in cardioids and the isolated cardiomyocytes. In cardiac tissue, we observed three specific bands in our cTnT Western blots. The fastest migrating band was present only in the cardioids, isolated cardiomyocytes, embryonic day 15 (E15) hearts, and neonatal hearts and was inversely related to the developmental state of the tissue. By contrast, the intensity of the ~40 KDa band increased with maturation. The ~40 KDa isoform observed here is likely the 41 KDa protein observed by Saggin et al. (28) in the adult heart, whereas the slower migrating form is the 42.5 KDa isoform they identified in the developing heart. The 23 KDa protein may represent an early developmental isoform that has yet to be identified in rat, or a proteolytic fragment resulting from the processing of the samples with lower total protein. Five isoforms of cTnT have been identified in the developing rabbit heart (29, 30), supporting the idea that this may represent a developmental isoform; however, more work would need to be done to confirm this in the rat. The very low levels of SERCA2a in the cardioids compared with the heart and 2-D cardiomyocytes suggest that progressive loss of SECRA2 occurs in our engineered tissue model. This observation may explain why engineered cardiac tissues become less spontaneously active and produce less force over time. If this is the case, interventions to maintain SERCA2 protein levels may be very important in the development of a functional cardiac replacement.

Isolated neonatal rat cardiomyocytes have provided a wealth of information on heart function since they were first isolated for in vitro study in 1964 (31). These studies have identified many important signaling pathways, as well as biochemical and electrophysiological properties of

cardiac cells. However, the lack of accompanying extracellular matrix makes it difficult to measure changes in cardiac function. Cardiac muscle functions to produce force. Without the capacity to measure force production, as is typically the case in a 2D culture system, other factors are measured as an end point. An example is the ongoing debate over cardiac hypertrophy. In isolated neonatal cardiomyocytes, where the specific force cannot be measured, an increase in protein-to-DNA ratio is often used as the correlate for hypertrophy. However, cardiac hypertrophy can be compensatory or decompensatory, depending upon whether specific force increases or decreases. For this reason, in vitro cardiac models that fail to include force production will continue to be inadequate for the study of many important characteristics of the heart as an organ. To this end we believe that functional engineered heart tissue will be a useful tool in basic cardiovascular research, and as a model of cardiac tissue development and repair.

## ACKNOWLEDGMENTS

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**Fig. 1**
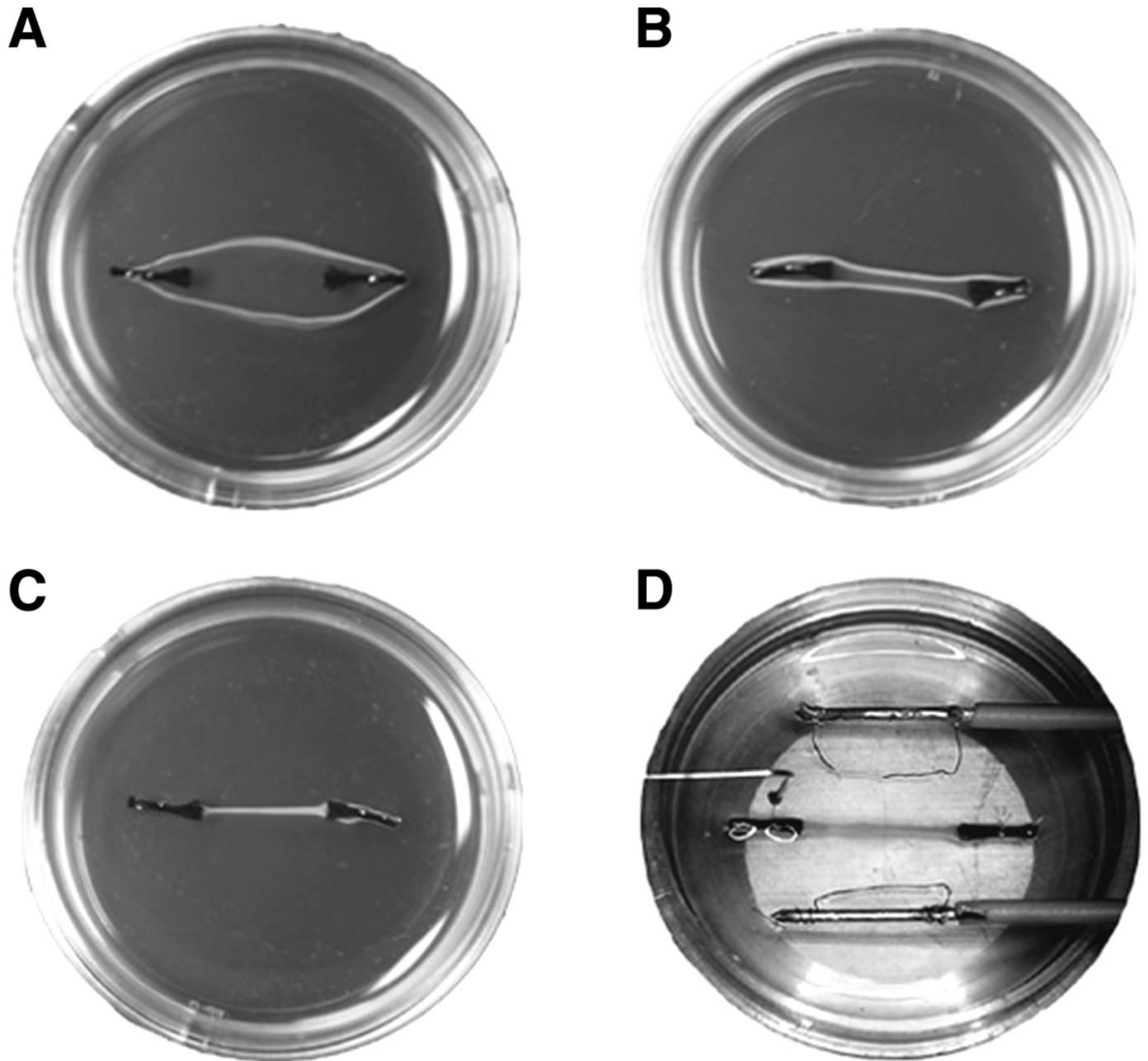


**Figure 1. The process of self-organization of cardiac muscle constructs in culture.** *A*) The cell monolayer begins to detach from the PDMS substrate at the periphery of the plate. *B*) Delamination of the monolayer progresses inward. *C*) The cell monolayer completely detaches from the substrate but remains attached at both anchor points, forming a three-dimensional self-organized tissue construct. *D*) The contractility of the tissue construct is evaluated by freeing one of the anchors, affixing a force transducer, and applying electrical stimulus pulses between parallel wire electrodes.

**Fig. 2**

**A**



**C**



**B**



**D**
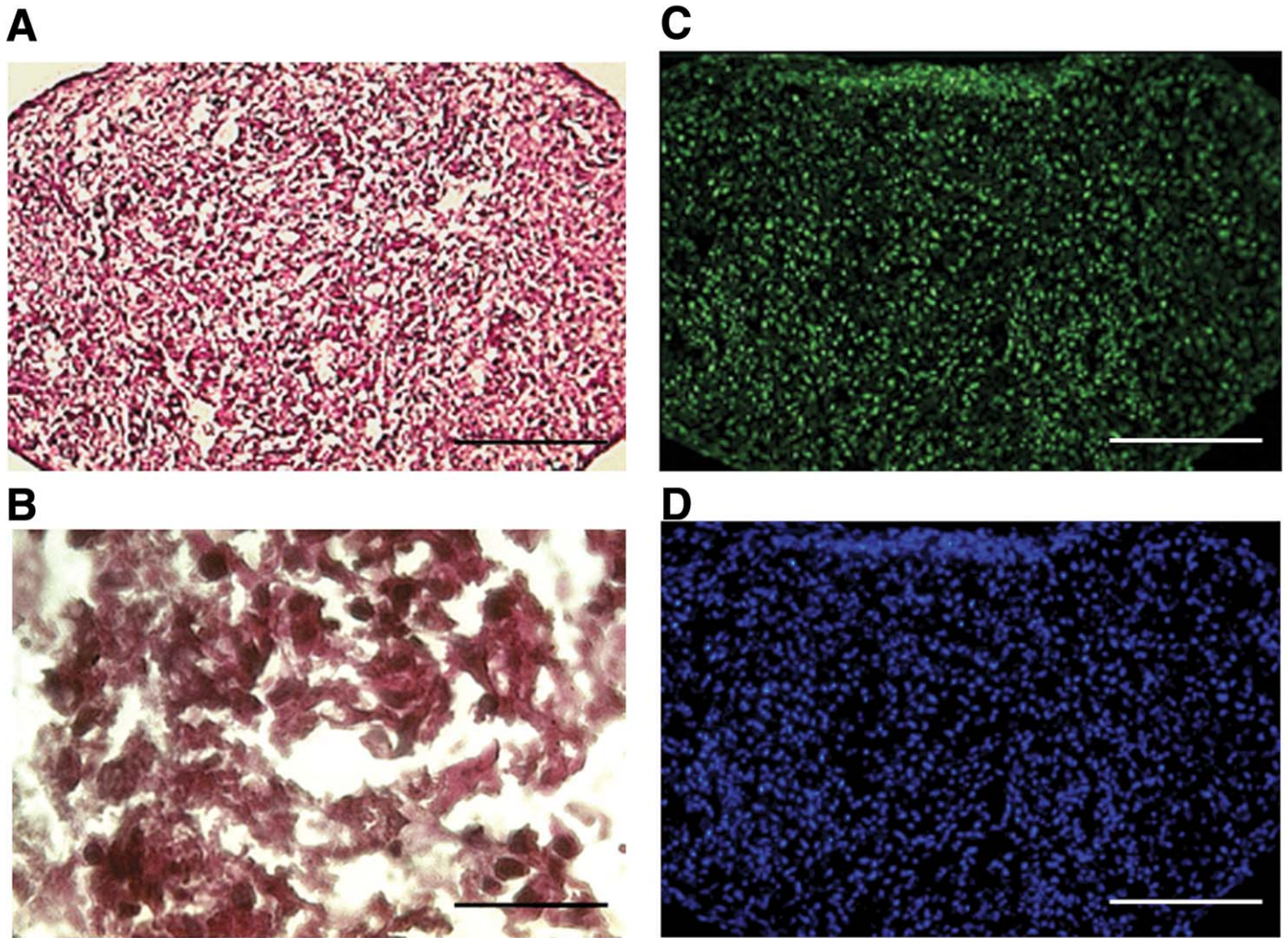


**Figure 2. Light micrographs of cardioids.** Representative light microscopic images of cardioids in cross section. Constructs were fixed, sectioned, and stained with: (*A*, *B*) hemotoxilin and eosin; (*C*) MF20 antibody (recognizing all isoforms of type II myosin heavy chain); and (*D*) DAPI (showing cellular nuclei). The bar in *A*, *C*, and *D* is 200 µm, whereas in *B* the bar represents 40 µm.

**Fig. 3**
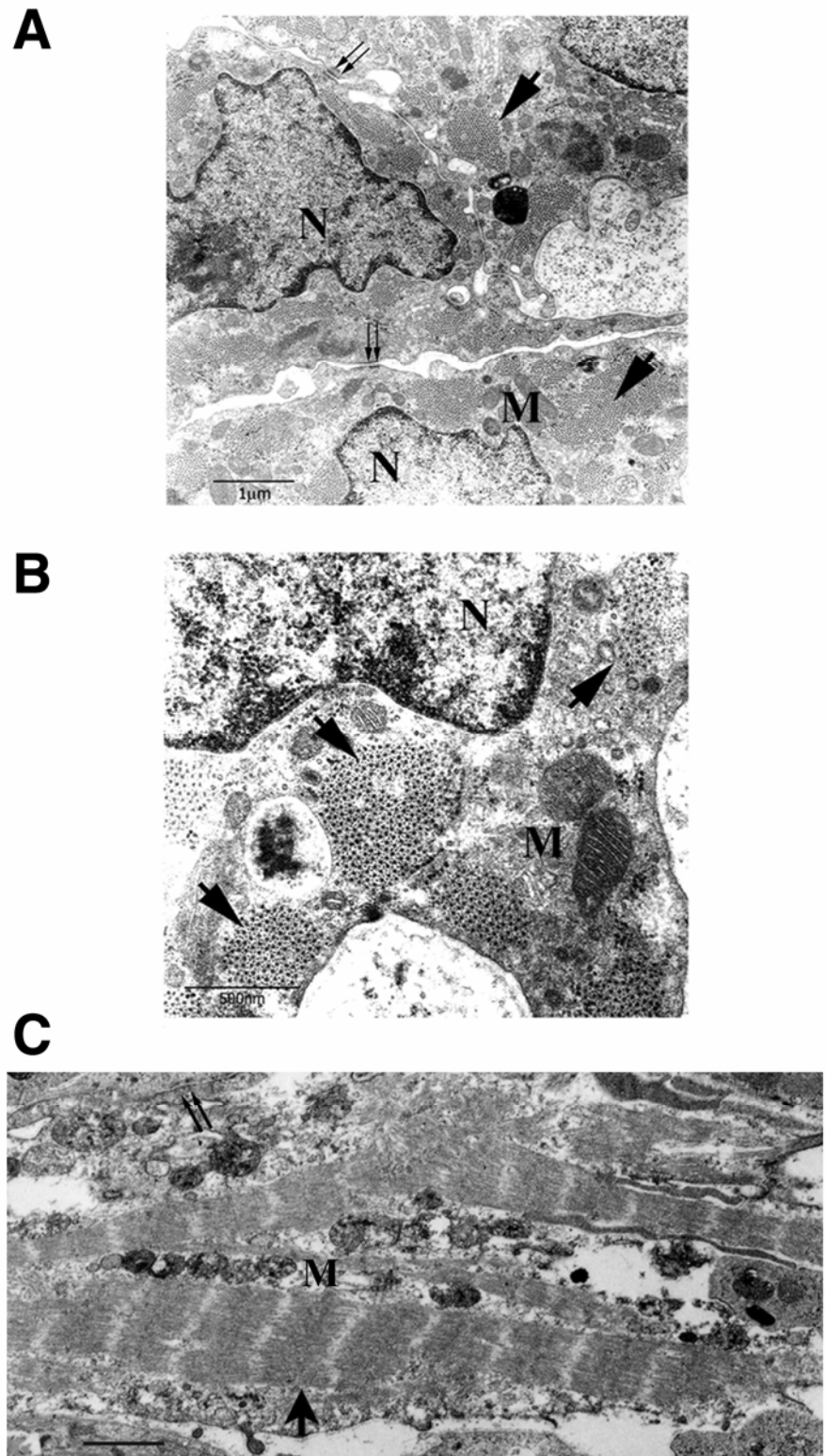


**Figure 3. Electron micrographs of cardioids.** Representative electron microscope images of cardioids in cross section show (*A*, *B*): hexagonally arrayed contractile proteins (⬆); areas rich in mitochondria (M) flanking the contractile machinery; a single prominent nucleus (N) in each cell; and gap junctions (↓↓) chemically linking the cells. In longitudinal sections (*C*), electron micrographs show: striated contractile proteins (⬆); subsarcolemmal mitochondria (M); and adherens junctions mechanically connecting the cells (↑↑). Reference length is given as a bar in each image: in *A* and *C* the bar represents 1 µm, while in *B* the bar is 0.5 µm.

**Fig. 4**
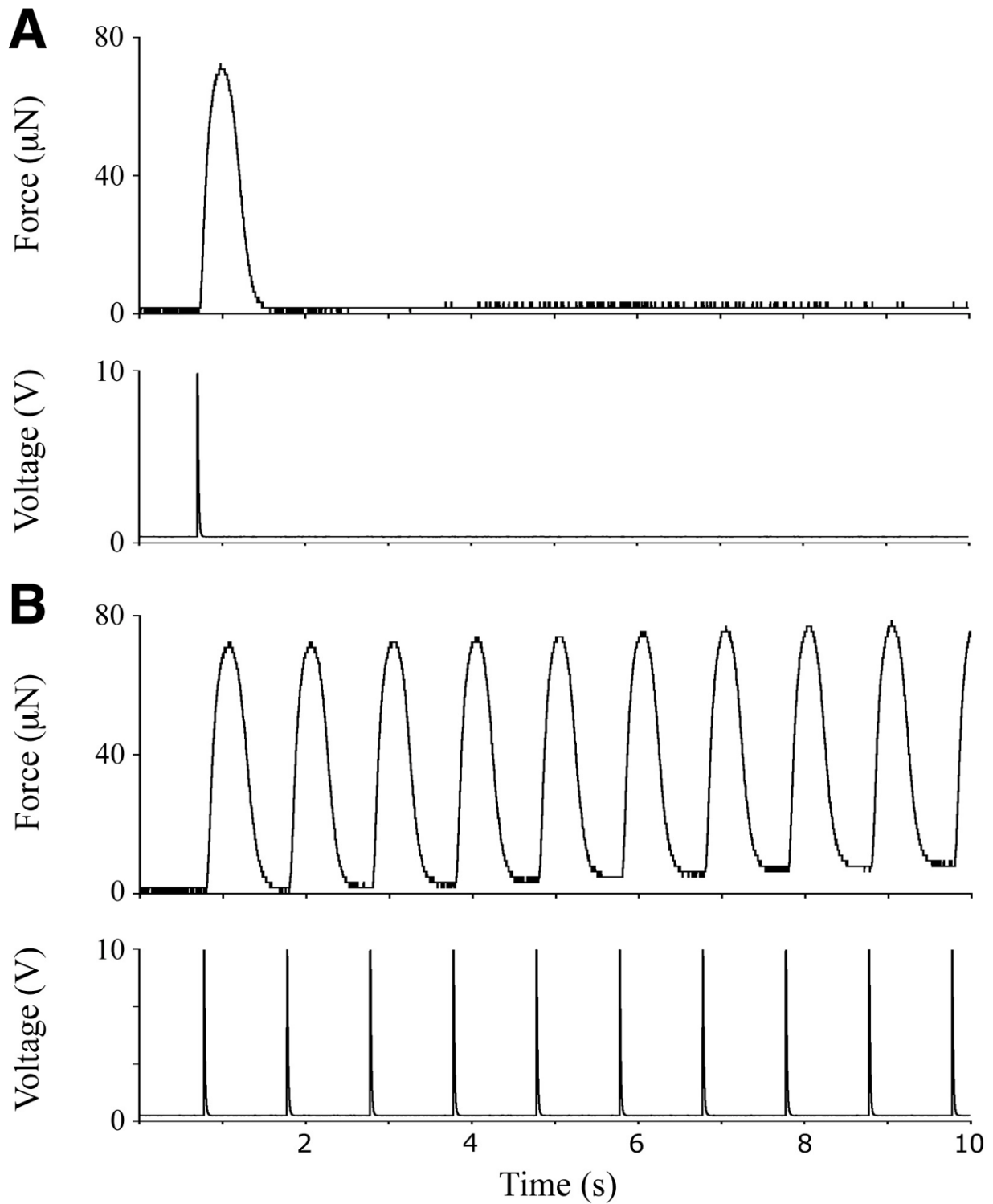


**Figure 4. Isometric contractility of 3-D engineered cardiac muscle.** Cardioid force production was determined following electrical stimulation. In all graphs, the top trace is force (in µN) as a function of time, while the bottom graph shows the electrical spikes used to stimulate the cardioids. *A*) A single 10 ms electrical pulse (10 V) is applied. *B*) A series of nine 10 V stimulation pulses are applied at a frequency of 1 Hz, the peak active force of the representative cardioid is ~70 µN.

**Fig. 5**
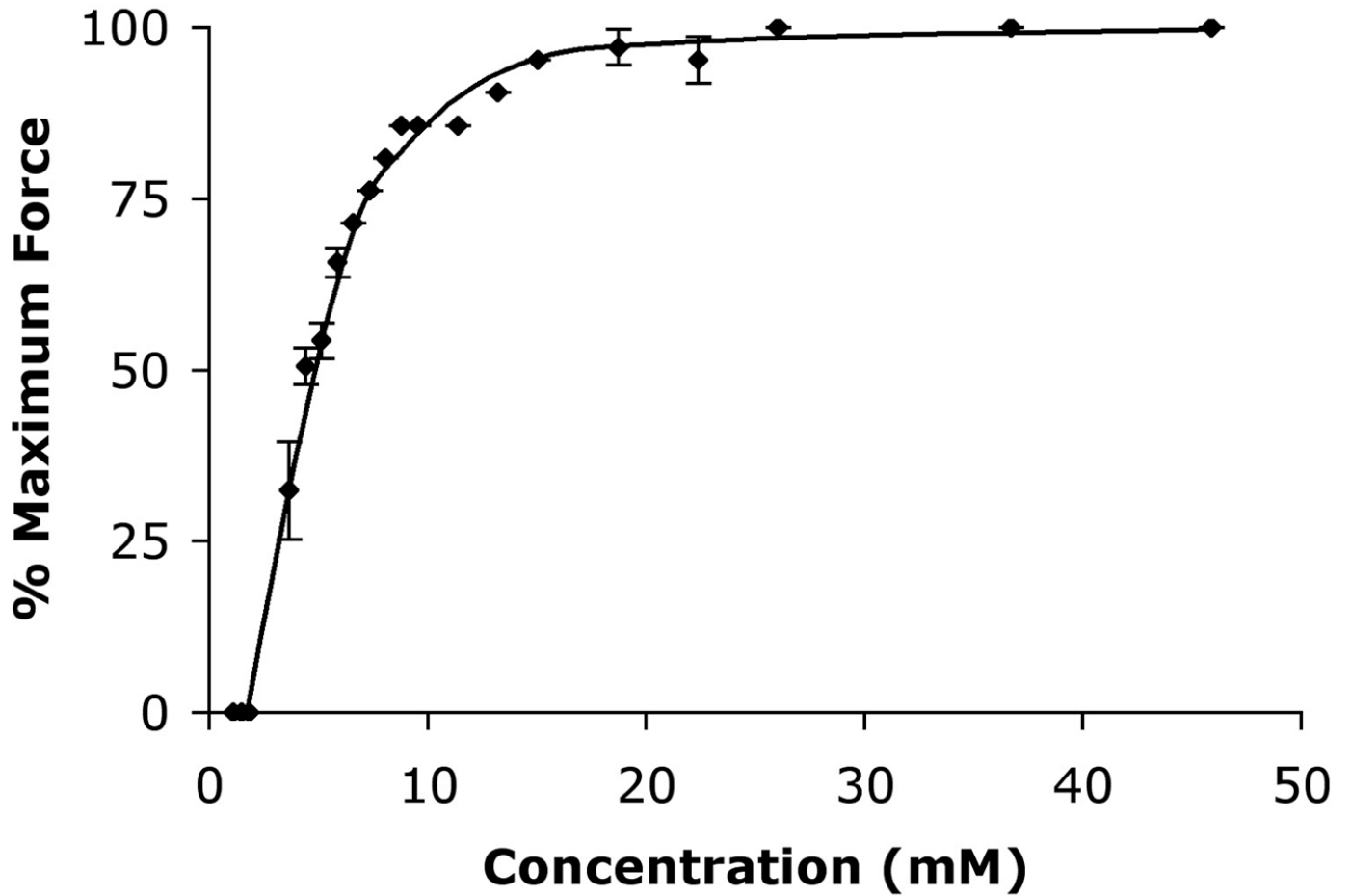


**Figure 5. Inotropic effects of calcium concentration.** Cardioids attached to a force transducer were electrically stimulated with a 10 V pulse lasting 10 ms in media containing from 0 to 50 mM calcium. Twitch force was recorded, and the percent maximal force was plotted as a function of calcium concentration. Trace is representative of data from six separate cardioids. Data are presented as mean ± SEM.

Fig. 6
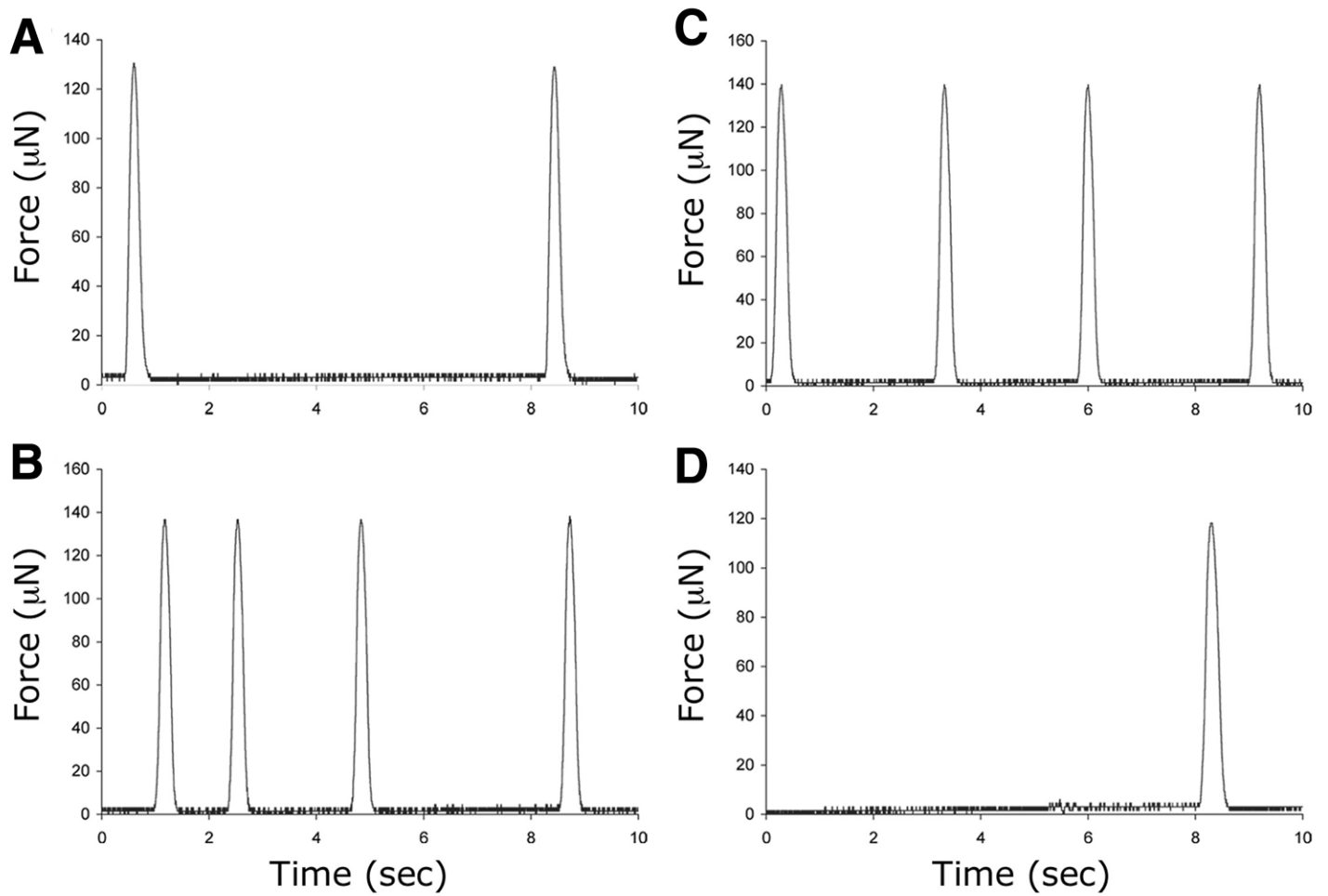
**Figure 6. Epinephrine increases the chronotropy of cardioids.** A representative series of traces showing the effect of increasing concentrations of epinephrine on the rate of spontaneous contraction of two-day-old cardioids. Measurements are shown from a single cardioid (**A**) before the addition of epinephrine, (**B**) after treatment with 0.4 µg or (**C**) 0.6 µg of epinephrine, and (**D**) following washout of the epinephrine and the addition of fresh media.

**Fig. 7**
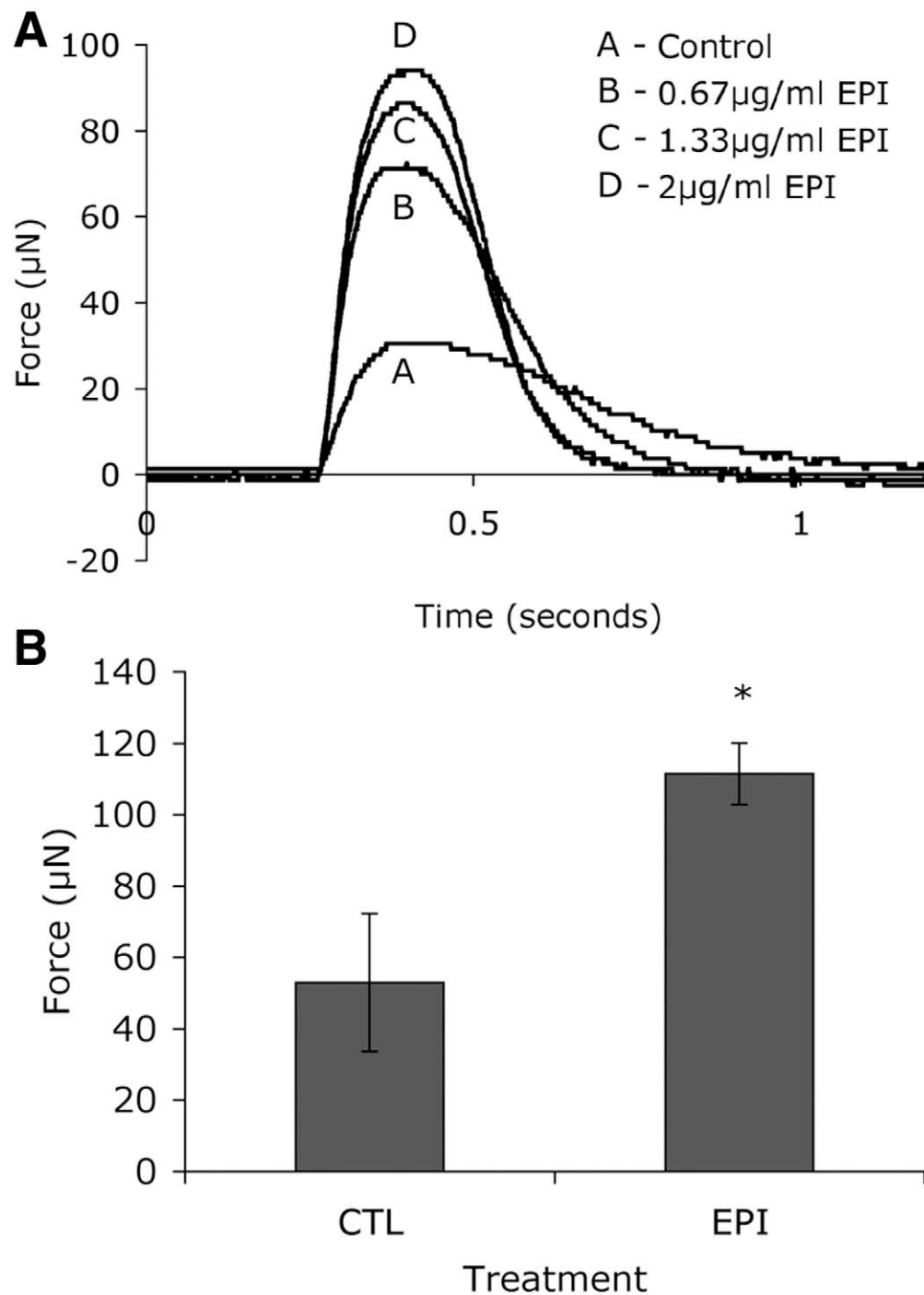


**Figure 7. Epinephrine increases cardioid inotropy and lusitropy.** Cardioids were electrically stimulated at 1 Hz for 10 s, and force response was measured. *A*) Representative traces of the second peak following the addition of increasing concentrations of epinephrine. Note that the relaxation of the contracting cardioid is more rapid as the concentration of epinephrine increases. The lusitropic effects of epinephrine peak at 1.33 µg/ml while the inotropic effects are maximal at 2 µg/ml (*B*) Quantification of the inotropic effect of maximal epinephrine on force production. The * represents a significant increase in force with the addition of epinephrine $P < 0.05$; $n = 4$ for each measurement.

**Fig. 8**
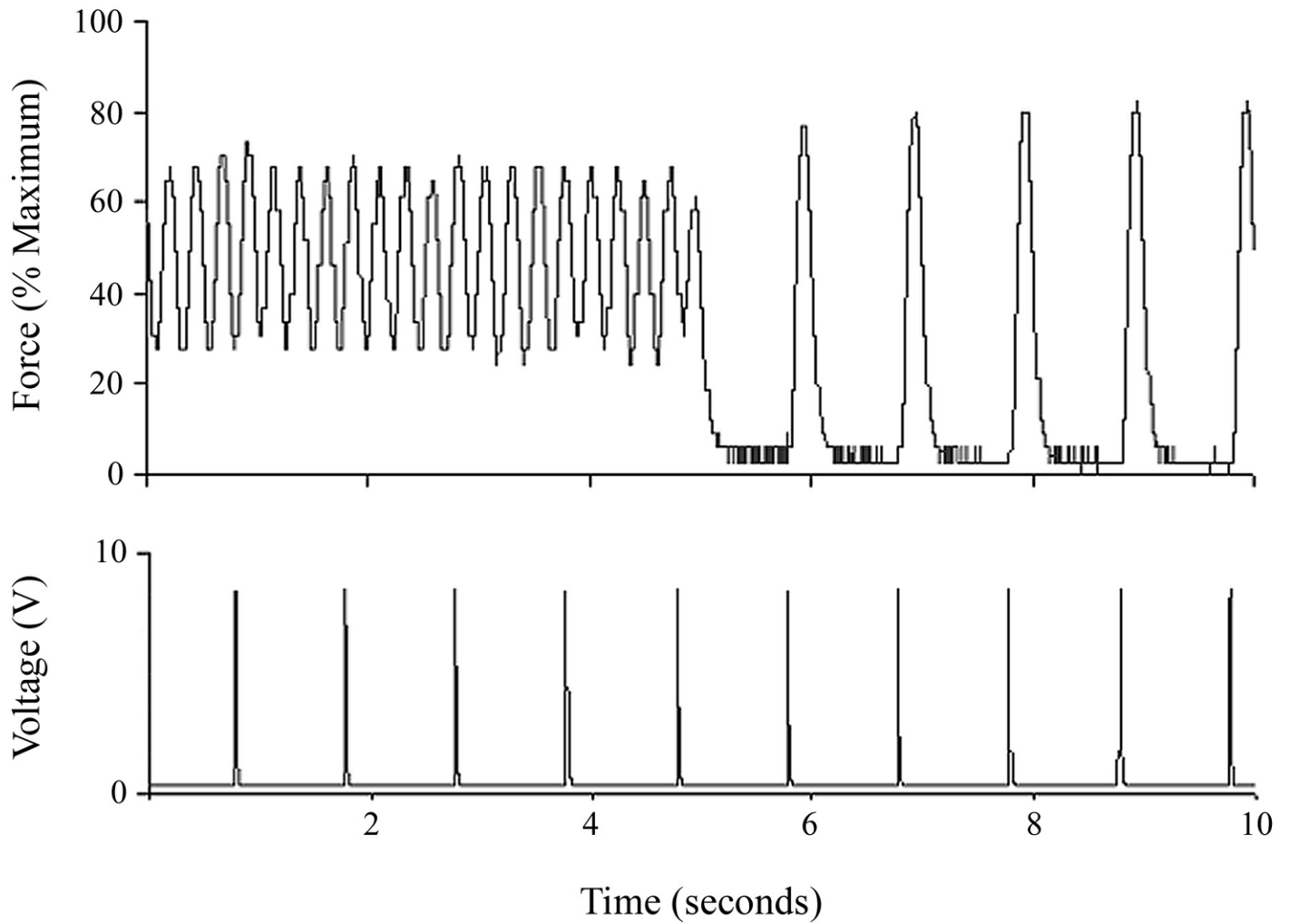


**Figure 8. Fibrillation of cardioids.** *A*) Cardioid force production determined as a function of time. Asynchronous spontaneous contractions of lesser magnitude (left half of *A*) until stimulated at the proper moment to induce refraction in the entire tissue. After this point, a regular train of contractions was produced in response to the 10V stimulation (right half of *A*). *B*) Stimulation trace for the cardioid. Traces are representative of more than 10 separate cardioids.

**Fig. 9**
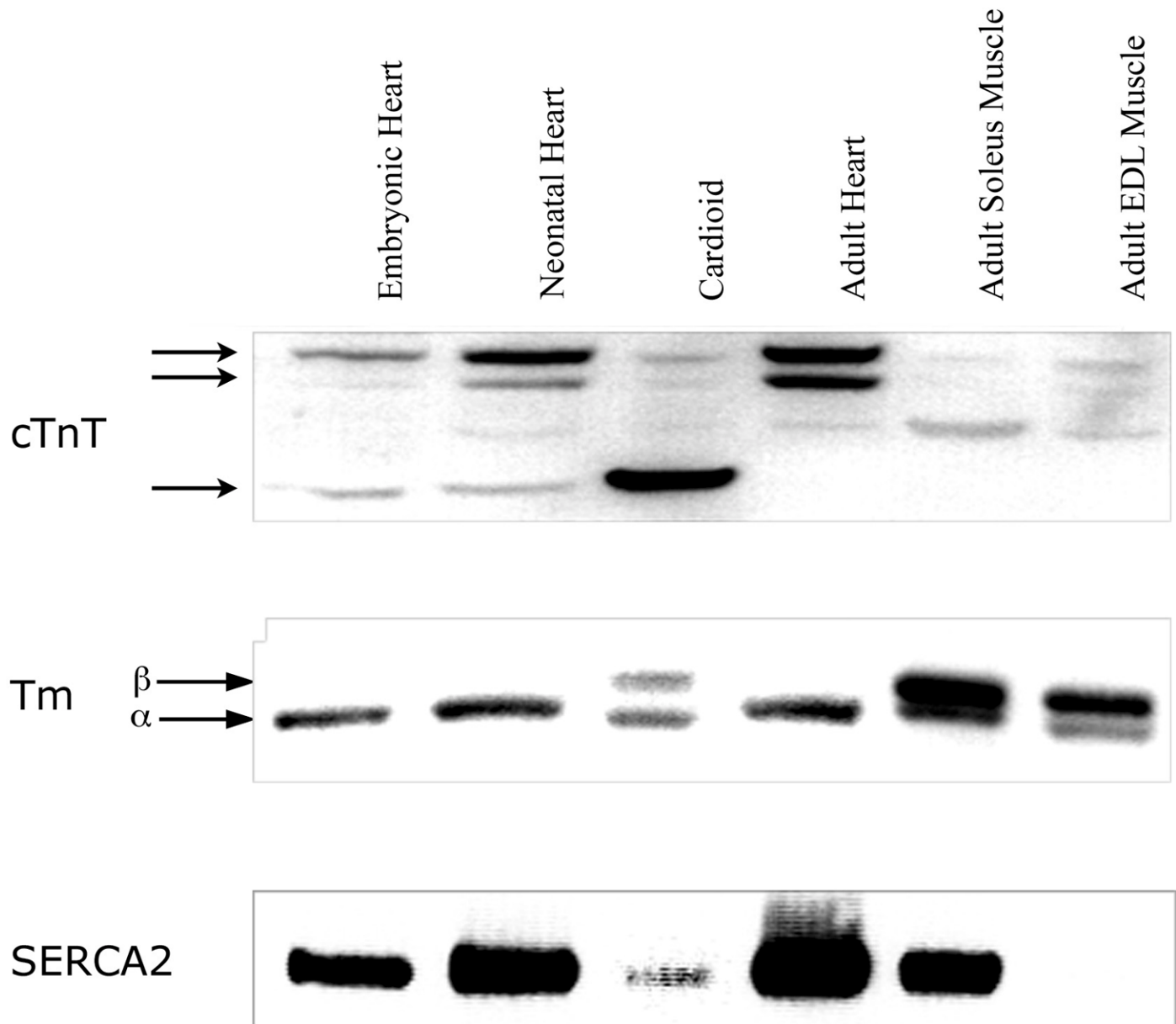


**Figure 9. Protein expression of cardioids is similar to early embryonic hearts.** *A*) Goat polyclonal antibody directed against cardiac troponin T (cTnT) recognized three primary bands in the cardiac tissue that were very low or absent from the skeletal muscles. *B*) Mouse monoclonal antibody directed against tropomyosin (donated by J. Metzger) recognized both the α and β forms of tropomyosin (Tm), α expressed in heart and β predominantly in skeletal muscle. *C*) Goat polyclonal antibody directed at SERCA2a recognizes a single band with a molecular weight of ~100 KDa in all of the heart tissues and slow skeletal muscle. As expected, SERCA2a is absent from the fast skeletal muscle sample. Images are representative westerns with minimum *n* = 8.

**Fig. 10**
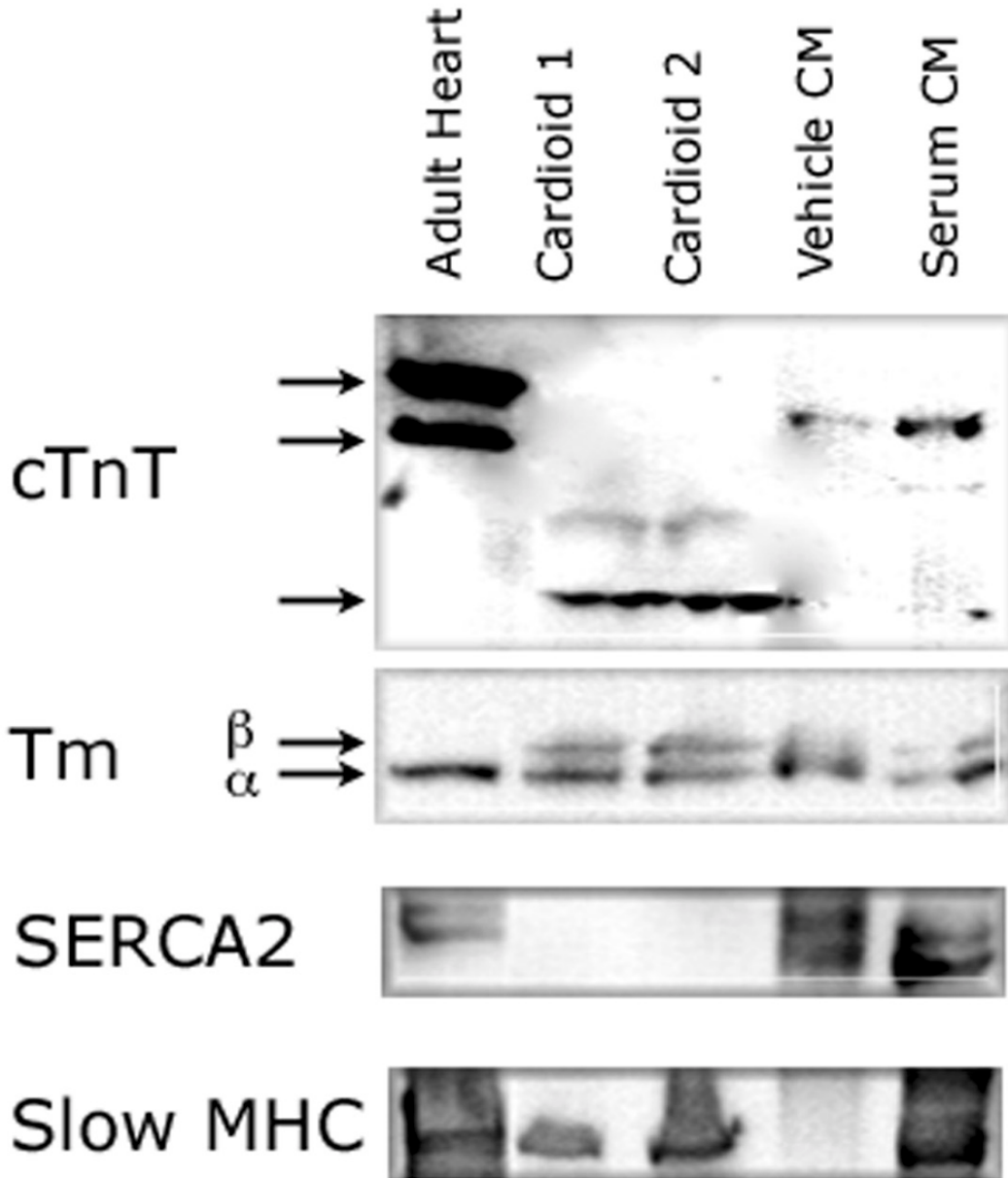


**Figure 10. Protein expression of cardioids compared with 2-D cultured cardiomyocytes.** cTnT, tropomyosin, SERCA2a, and slow/cardiac myosin heavy-chain protein in 18-day-old cardioids compared with the adult heart (far left) and four-day cultures of isolated neonatal cardiomyocytes (two right lanes). The cultured neonatal cardiomyocytes express higher levels of SERCA2a and express low levels of the ~23 KDa isoform of cTnT. Images are representative westerns with minimum $n = 4$.