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

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SPECIFIC AIMS

Engineering 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, engineered cardiac muscle could be used to develop replacement tissue for individuals with heart failure. Furthermore, directing neonatal cardiomyocytes to self-organize into contractile cardiac tissue may yield valuable information on essential developmental processes.

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. We designate these tissue constructs *cardioids*, as they are similar to cardiac muscle in terms of cell-to-cell connectivity and contractility.

PRINCIPAL FINDINGS

1. A monolayer of cardiac cells will delaminate and self-organize into a 3-D tissue

Within 1 wk of plating, a monolayer of cardiac cells, including cardiomyocytes and cardiac fibroblasts, had developed and begun contracting as a syncytium. Approximately 180 h after plating, the monolayer of cells began to detach from the periphery of the laminin substrate. At its peak rate, the process of self-organization from a monolayer into a cylindrical 3-D structure was visible to the naked eye. 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. 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 anchors at each end. 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.

2. Cardioids have cardiac muscle-like ultrastructure

Light micrographs stained with the MF20 antibody, which recognizes all isoforms of type II myosin heavy chain, and counterstained with DAPI to show all nuclei showed that >70% of the cells expressed the myosin heavy chain and were likely cardiomyocytes. Electron micrographs showed organized contractile units and early z-line structures in longitudinal images (**Fig. 1*C***). In cross section (Fig. 1*B*), hexagonally arrayed myofilaments were visible. As in 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 were adherens junctions that serve to mechanically link cardiomyocytes. A large number of gap junctions were evident on the lateral surfaces of the cardiomyocytes (Fig. 1*A*). These structures serve as a chemical linkage between the cells and may be important in electrical coupling of the cardiomyocytes.

3. Cardioids contract like cardiac tissue

Forty-eight hours after delamination was completed, 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. At given calcium concentrations, both spontaneous and induced contractions resulted in identical peak force production. However, positive inotropy was

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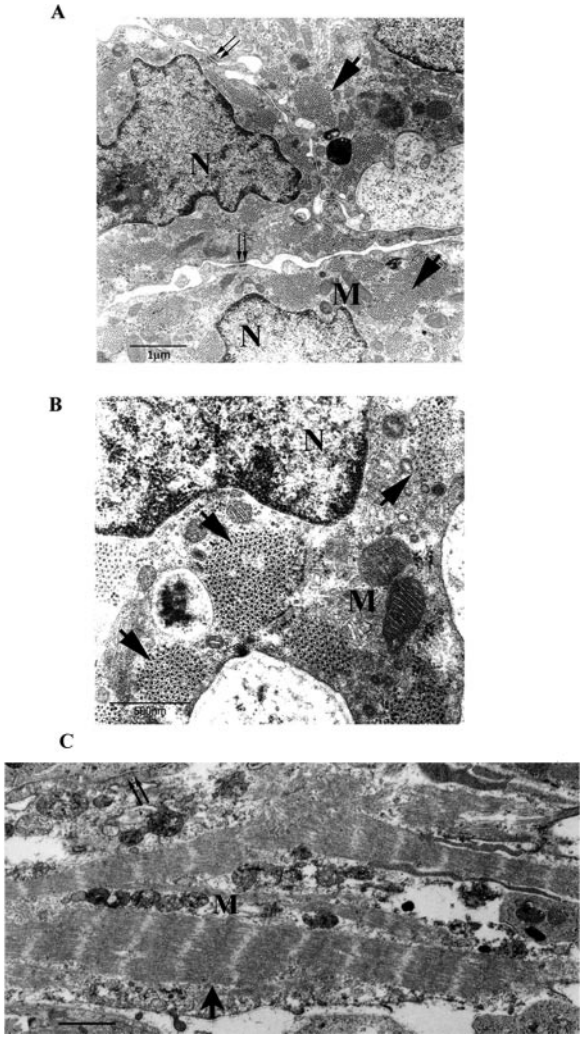

Figure 1. Electron micrographs of cardioids. Representative electron microscope images of cardioids in cross section show *A, B*) hexagonally arrayed contractile proteins (□), areas rich in mitochondria (M) flanking the contractile machinery, a single prominent nucleus (N) in each cell, and gap junctions (↓ ↓) 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: *A, C)* bar = 1 μm; *B)* bar = 0.5 μm.

observed with increasing calcium concentration in the media.

The cardioids could be entrained to contract with a "pacemaker" set to 1 Hz for an indefinite period without detectable fatigue. By comparison, engineered skeletal muscle constructs require 30 to 120 s between peak twitches to once again be able to produce an equivalent peak twitch force. 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 ± 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.

To test the contractile response to cardioactive drugs, cardioids were stimulated in the presence of epinephrine. Epinephrine treatment increased the rate of spontaneous contraction in a dose-dependent manner and induced a dose-dependent response for inotropy and lusitropy when stimulation occurred in serum-free media containing a half-maximal concentration of calcium (**Fig. 2**). The force produced by the cardioids treated with maximal epinephrine increased 253 ± 58% while half-relaxation time decreased 32 ± 14%.

4. Cardioids have an early embryonic protein phenotype

To determine the developmental state of the cardioids, Western blots were performed to study the isoforms of tropomyosin, SERCA2a, and cardiac troponin T (cTnT). The cardioids were phenotypically similar to early developmental cardiac muscle. Four 4 days after delamination, cardioids express both α and β-tropomyosin. Muthuchamy et al. have shown that at the earliest

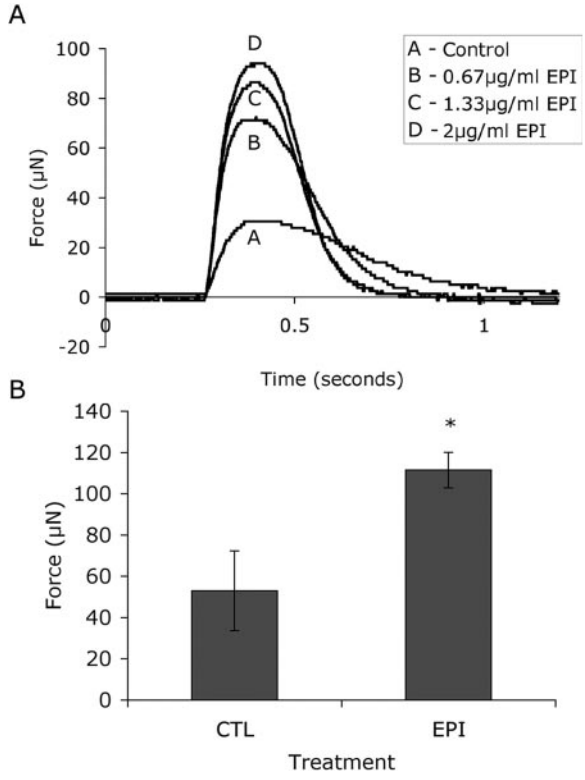

Figure 2. Epinephrine increases the inotropy and lusitropy of cardioids. 2-Day-old cardioids were electrically stimulated at 1 Hz for 10 s and the force response was measured. *A)* Representative traces of the second peak after the addition of increasing concentrations of epinephrine. Note that relaxation of the contracting cardioid is more rapid as the concentration of epinephrine increases. Lusitropic effects of epinephrine peak at 1.33 μg/mL; inotropic effects are maximal at 2 μg/mL. *B*) Quantification of the inotropic effect of maximal epinephrine on force production. *Significant increase in force with the addition of epinephrine $P < 0.05$.

developmental state of mouse cardiac tissue, α and β tropomyosin are expressed, consistent with what we observe in cardioids. Expression of cTnT followed a similar pattern. In cardiac tissue, we observed three specific bands. The fastest migrating band at ~23 kDa was present only in the cardioids, embryonic day 15 (E15) hearts, and neonatal hearts and was inversely related to the developmental state of the tissue. The intensity of the ~40 kDa band increased with maturation and is likely the 41 kDa protein observed by Saggin et al. in the adult heart, while the slower migrating form is the 42.5 kDa isoform they identified in the developing heart. Levels of cardiac/slow skeletal SERCA2a were low in the E15 heart, increased in the neonatal, and high in the adult heart and slow skeletal muscle. The cardioids produce a low but detectable level of SERCA2a. This is consistent with work by Liu et al. demonstrating that SERCA2a was barely detectable in 9.5 day postcoital (d.p.c.) mouse hearts. They showed that the level of SERCA2a increased ~10-fold by 18 d.p.c. and a further 2-fold in the adult.

Cardiomyocytes collected 4 days after plating express high levels of α and β-tropomyosin, show the ~23 kDa cTnT band, and have high levels of cardiac MHC; 4-day-old cells have significantly higher SERCA2a than cardioids. This suggests a loss of SERCA2a in the cardioids and may explain why engineered cardiac tissues become less spontaneously active and produce less force over time. If so, interventions to maintain SERCA2 protein levels may be important in the development of a functional cardiac replacement.

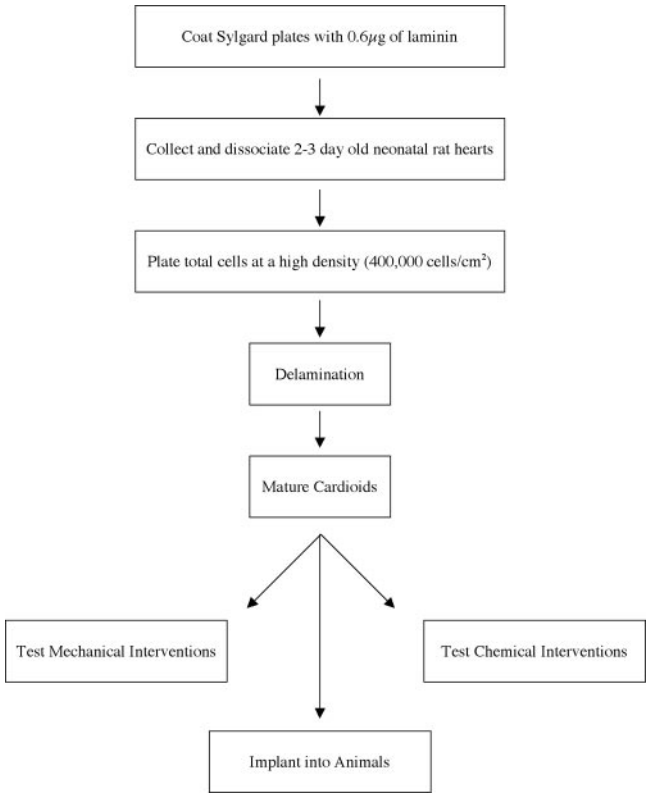

Figure 3. Schematic diagram of the development of cardioids. Hearts are removed from 3-day-old rat pups, dissociated, and formed into 3-D cardiac tissue in vitro. These "cardioids" are phenotypically similar to developing hearts, suggesting they may be a good model for studying cardiac development in vitro.

CONCLUSIONS AND SIGNIFICANCE

Conditions have been identified that promote the formation of self-organizing contractile cardiac tissue from disaggregated neonatal rat cardiac cells. The 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 myofibrillar 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, then pour the cells into molds of various sizes and shapes. After 7 days in culture, EHTs are placed in a bioreactor that applies a unidirectional stretch (10% stretch at a frequency of 2 Hz). Mechanical stretch promotes the alignment of cardiomyocytes within the gel and increases the size of the cells as well. While the cyclic loading promotes the development of EHT, their normalized force is relatively small, in the range of 2.1–4.3 kN/m^2, compared with the 67 ± 30 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. FJ