Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies

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The feasibility of engineering functional cardiac muscle has been previously demonstrated by others (1, 11) and ourselves (5, 6, 14). Eschenhagen et al. (11) demonstrated that embryonic chick cardiac myocytes cultured in collagen gels displayed characteristic physiological responses to physical and pharmacological stimuli, and Akins et al. (1) showed that rat ventricular cardiomyocytes cultured on polystyrene microcarrier beads in bioreactors formed 3D spontaneously contrac tile aggregates. Our group reported that cultivation of neonatal rat cardiac myocytes on polyglycolic acid (PGA) scaffolds in bioreactors resulted in contractile 3D tissues (14) with ultrastructural features and electrophysiological properties characteristic of cardiac muscle (5), and we provided evidence that variations in initial cell density and cultivation conditions affected the structure of engineered cardiac tissue (6).

The goals of the present study were to further improve the structure and function of engineered cardiac muscle and to correlate specific molecular parameters with electrophysiological function. We hypothesized that molecular, structural, and electrophysiological properties of engineered cardiac tissue were interrelated and could be varied and improved by changing specific model system parameters. The specific aims were to: 1) use semiquantitative molecular biological methods to assess gap junctional protein (connexin43; Cx43) (31) and two other markers of differentiated muscle [creatine kinase isoform-MM (CK-MM) and sarcomeric myosin heavy chain (MHC)] (18, 22); 2) relate the expression of molecular markers to tissue structure (histomorphology and composition) and electrophysiological function; and 3) investigate the sensitivity of tissue properties to controlled variations in model system parameters. In particular, cultivation in rotating bioreactors was studied as an alternative to mixed flasks, because the former provides a unique, low-shear environment that has been associated with improved in vitro development of engineered cartilaginous (45) and cardiac-like tissues (6) and enhanced expression of differentiated phenotype by a wide range

Because of the limited regeneration potential of cardiac myocytes (8), attempts to improve the functionality of the heart after a myocardial infarction have involved the injection of dissociated myogenic cells into the scarred myocardium (34, 39, 46). It has been proposed that implantation of functional, in vitro-grown cardiac muscle instead of isolated cells could potentially improve cell localization and the efficiency of tissue repair (5, 6). Three-dimensional (3D) engineered cardiac muscle could also provide a model system for basic cardiovascular research (1, 5, 6, 11, 14, 15).

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of cell types (41). Low-serum media were studied as an alternative to high-serum media in an attempt to enhance cardiac myocyte differentiation (25, 33, 40) and suppress proliferation of nonmyocytic cells (30). Laminin-coated scaffolds were studied as an alternative to uncoated ones to promote cell attachment and differentiation, as previously reported for monolayer cultures of cardiac myocytes (4, 10, 20).

**MATERIALS AND METHODS**

All experiments involving animals were performed according to a protocol approved by the Massachusetts Institute of Technology Committee on Animal Care, which follows federal and state guidelines.

**Materials**

All materials were purchased from Sigma unless otherwise specified.

**Media**

Two different media formulations were used: 1) high-serum medium (Dulbecco’s modified Eagle’s medium (DMEM; GIBCO-BRL) with 4.5 g/l glucose, 10% fetal bovine serum (FBS; Hyclone), 10 mM HEPES (GIBCO-BRL), 4 mM L-glutamine (GIBCO-BRL), and 100 U/ml penicillin (GIBCO-BRL)) and 2) low-serum medium (DMEM with 4.5 g/l glucose, 2% adult horse serum (Hyclone), 10 mM HEPES, 4 mM L-glutamine, and 100 U/ml penicillin).

**Cells**

Primary cultures of cardiac myocytes were prepared by digesting ventricles obtained from neonatal (2 day old) Sprague-Dawley rats (Taconic) with 0.06% trypsin (GIBCO-BRL) and 0.1% collagenase type II ( Worthington) followed by two preplatings, 1 h each, to increase the fraction of cardiac myocytes (5, 6). The resulting cell population was expected to contain >80% myocytic cells and <20% nonmyocytic cells, as previously reported (1, 26). Isolated cells were resuspended in high-serum medium. The cell yield was ~6 x 10^6 cells/ventricle; cell viability was 91 ± 3% as assessed by trypan blue exclusion. A total of 160 ventricles (16 liters) were used in four independent experiments.

**Scaffolds**

The basic scaffold, a PGA mesh formed as 5-mm-diameter, 2-mm-thick disks, has been previously characterized (16). In brief, PGA was extruded into 13-μm-diameter fibers that were processed into a nonwoven mesh with a porosity of 97%. Two modifications of the basic scaffold were studied: 1) surface-hydrolyzed PGA mesh (sPGA) made by incubating PGA in 1 N NaOH for exactly 1 min at room temperature, rinsing with deionized water for 30 s, washing with 95% ethanol alcohol and ether for 1 min each, lyophilizing, and sterilizing with ethylene oxide (17); and 2) laminin-coated sPGA mesh (lsPGA) made using laminin derived from Engelbreth Holm Swarm mouse sarcoma (1 scaffold and 0.1 ml of 5 μg/ml laminin were incubated at 37°C for 4 h in a 96-well plate on an orbital shaker set at 60 rpm) followed by lyophilization and ethylene oxide sterilization. Laminin-coated scaffolds were prepared immediately before use to minimize laminin degradation.

**Cell Seeding and Cultivation Vessels**

Three culture vessels were used, and all were operated in a 37°C humidified 5% CO₂ incubator: 1) 96-well plates that contained 0.06 ml of media and one scaffold per well and were mixed at 25 rpm using an XYZ gyrator (Boeker Scientific), 2) spinner flasks (Belloco) that contained 120 ml of media and six scaffolds fixed to stainless steel wires embedded in the stopper and were magnetically stirred at 50 rpm (45), and 3) rotating vessels (the high aspect ratio vessel (HARV) from Synthecon) that contained 100 ml of media and six scaffolds that were freely suspended by vessel rotation as a solid body at 12 rpm (6, 14, 15). Each HARV (a cylindrically shaped vessel 10 cm in diameter and 1.3 cm high) was mounted on a base that provided rotation about the central axis of the vessel and pumped incubator air over its internal gas exchange membrane (78.5-cm² surface area).

**Cell seeding studies.** PGA (n = 17) and sPGA (n = 17) were compared by pipetting a concentrated cell suspension onto the scaffolds in 96-well plates (0.06 ml of 1.33 x 10^6 cells/ml per scaffold, 1 scaffold per well) and incubating the plates for 1 h on an XYZ gyrator operated at 25 rpm. The duration of seeding on XYZ gyrators was limited to 1 h to minimize cell death, which increased significantly by 2 h (as was shown in preliminary studies). Additional studies were done to compare two cell concentrations and two seeding methods by forming three groups. In the first group (n = 16), a concentrated cell suspension was pipetted onto sPGA in 96-well plates (0.06 ml of 1.33 x 10^6 cells/ml per scaffold, 1 scaffold per well) and the plates were incubated on an XYZ gyrator operated at 25 rpm for 1 h. Cell polymer constructs and the remaining unseeded cells were then transferred into 100 ml HARVs and rotated at 12 rpm for an additional 1 h. In the second group (n = 16), a concentrated cell suspension was pipetted onto sPGA scaffolds fixed to needles in spinner flasks (0.06 ml of 1.33 x 10^6 cells/ml per scaffold, 6 scaffolds per flask), and the flasks were g yrated for 1 h at 25 rpm. Flasks were then filled with 100 ml of medium and stirred at 50 rpm for an additional 1 h. In the third group (n = 16), cells were directly inoculated into HARVs at a lower concentration (100 ml of 4.8 x 10^6 cells/ml, 6 sPGA per HARV) and rotated for 2 h. In all of the above cell seeding studies, constructs sampled after 1–2 h were compared with respect to DNA content, measured as described below.

**Tissue cultivation studies.** The effects of cultivation vessel, medium composition, and tissue culture substrate on engineered cardiac tissue properties were assessed by forming four experimental groups as follows. In the sPGA/flask/high-serum group (n = 10), a concentrated cell suspension was pipetted onto sPGA fixed to needles in a flask (0.06 ml of 1.33 x 10^6 cells/ml per scaffold, 6 scaffolds per flask), and the flask was g yrated for 1 h at 25 rpm. High-serum media (100 ml) was then added, and flasks were cultivated for 1 wk with mixing at 50 rpm. Medium was replaced by 100% on culture day 2 and by 50% on days 4 and 6. In the sPGA/HARV/high-serum group (n = 10), a concentrated cell suspension was pipetted onto sPGA in 96-well plates (0.06 ml of 1.33 x 10^6 cells/ml per scaffold, 1 scaffold per well), and plates were g yrated for 1 h at 60 rpm. Cell-polymer constructs and the remaining unseeded cells were then transferred into HARVs containing 100 ml of high-serum media and cultivated for 1 wk at 12 rpm. Medium was replaced by 100% on day 2 and by 50% on days 4 and 6. The sPGA/HARV/low-serum group (n = 10) was identical to the sPGA/HARV/high-serum group except that on day 2 the culture was switched to low-serum media and continued for 5 more days, replacing the media by 50% on days 4 and 6. The lsPGA/HARV/low-serum group (n = 10) was identical to the sPGA/HARV/low-serum group except that laminin-coated sPGA was used.
Native Tissues

To verify the analytic methods, evaluate the developmental state of cardiac myocytes in engineered tissues, and establish baseline values for parameters not readily found in the literature, ventricular tissue was obtained from neonatal (2 day old) rats (n = 10 from 3 litters) as previously described (5). In brief, tissue samples weighing 7–13 mg were used for histological, biochemical, and molecular analyses, whereas full-thickness pieces of the anterior ventricular wall (~6 × 4 × 2 mm) were used for electrophysiological studies.

Analytic Methods

Media analyses. Cell damage and death during seeding and cultivation were assessed by measuring lactate dehydrogenase (LDH) levels (n = 3 samples per time point) in sonicated media using a commercial kit (Chiron). Media concentrations of glucose and lactate (n = 3 samples per time point) were measured using a glucose and lactate analyzer (model 2300 STAT Plus, Yellow Springs Instruments).

Light microscopy. One-week constructs were fixed in 2% glutaraldehyde for 10 min, rinsed in PBS, fixed for 24 h in 10% neutral-buffered formalin (NBF), embedded in paraffin, sectioned (5 µm thick), and either stained with hematoxylin and eosin (H-E) or immunostained with a monoclonal antibody to sarcomeric tropomyosin as previously described (5). The fraction of cardiac myocytes (i.e., the fraction of cells that stained positively for sarcomeric tropomyosin) was assessed in six different samples using 10 randomly selected (0.3 mm × 0.4 mm) fields per sample by videomicroscopy and the National Institutes of Health Image 1.60 software.

Immunofluorescence microscopy. Laminin coating of sPGA fibers was verified using an antibody to laminin in conjunction with confocal microscopy. Briefly, sPGA or sPGA scaffolds were fixed in 10% NBF for 30 min, rinsed with PBS, blocked with horse serum for 30 min and incubated with a rabbit anti-laminin polyclonal antibody diluted 1:60 in PBS containing 0.5% Tween 20 and 1.5% horse serum (PBS-T20) for 1 h at room temperature. Scaffolds were extensively washed in PBS and incubated with a fluorescein-conjugated goat anti-rabbit IgG diluted 1:100 in PBS-T20 for 1 h in the dark, placed on glass slides, and coverslipped using glycerol mounting media. The absorbed laminin on the sPGA fibers was visualized at glass slides, and coverslipped using glycerol mounting media. IgG diluted 1:100 in PBS-TH for 1 h at room temperature. Scaffolds were extensively washed in PBS with a fluorescein-conjugated goat anti-rabbit IgG diluted 1:100 in PBS-TH for 1 h in the dark, placed on glass slides, and coverslipped using glycerol mounting media.

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Transmission electron microscopy. Samples for transmission electron microscopy were fixed in Karnovsky’s reagent (0.1 M sodium cacodylate with 2% paraformaldehyde and 2.5% glutaraldehyde, pH 7.4), postfixed in 1% osmium tetroxide in veronal-acetate buffer, dehydrated in graded ethanol in propylene oxide, and embedded in Epon 812 (Polysciences). Sections (70 nm thick) were prepared using a Leica Ultra Cut and a diamond knife, stained with lead citrate and uranyl acetate, and examined using a Philips EM410 transmission electron microscope operated at 80 kV.

Biochemical and molecular assays. Samples for DNA, total protein, and Western blot analyses were homogenized in buffer (1 N NH₄OH/2% Triton X-100, 0.04 ml/mg wet wt of sample) for 1 min. DNA was measured fluorometrically by Hoechst binding, and total protein was measured by a commercially available kit (Bio-Rad) as previously described (5, 6). To quantify the relative expression levels of Cx43, MHC, and CK-MM, a cocktail of protease inhibitors [0.5% SDS, 50 mM Tris-HCl (pH 7.4), 1 mg/ml leupeptin, 1 mg/ml pepstatin, 0.1 M phenylmethylsulfonyl fluoride, and 1 mg/ml aprotinin] was added to the homogenate. After centrifugation for 10 min at 12,000 g at 4°C, the samples were stored at −80°C. On the day of analysis, a subset of the homogenates were treated with 4 U/ml alkaline phosphatase (Boehringer-Mannheim) for 4 h at 37°C to study the phosphorylation patterns of Cx43. Homogenates were diluted (1 part sample to 2 parts buffer) in Tricine (for Cx43 and CK-MM) or Laemmli (for MHC) buffer (Bio-Rad) containing 5% mercaptoethanol and boiled for 5 min. Homogenates containing 15 µg of total protein each were separated on 10–20% Tris-Tricine (for Cx43 and CK-MM) or 4–15% Tris-glycine (for MHC) minigels (Bio-Rad) using kaleidoscope prestained standards (Bio-Rad) at a constant voltage of 100 V for 2 h at room temperature. Purified human CK-MM (Biodesign), bovine myosin, and rat brain lysate (Transduction Laboratories) were used as positive controls for CK-MM, MHC, and Cx43, respectively. To compare the intensity of the protein bands between two gels, both gels were loaded with the same experimental sample in triplicate.

ELuted proteins were electrophoretically run in 1× Tris/Tricine/SDS (for Cx43 and CK-MM) or Tris/glycine/SDS (for MHC) running buffer (Bio-Rad) onto polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 60 min at room temperature in
a Bio-Rad Trans-Blot cell. Blots were first incubated overnight at 4°C with 5% nonfat dry milk in PBS-T to block nonspecific binding of antibodies and then, for an additional 1–2 h, with the appropriate primary antibody. The primary antibodies were 1) rabbit anti-Cx43 (Zymed), diluted 1:1,000 in PBS-T; 2) goat anti-CK-MM (Biodesign), diluted 1:2,500 in PBS-T; and 3) mouse anti-MHC (Developmental Studies Hybridoma Bank), diluted 1:100 in PBS-T. Blots were washed five times with PBS-T and incubated for 1 h at room temperature with sheep anti-mouse, rabbit anti-goat, or sheep anti-rabbit IgG antibodies (Amersham), respectively, all conjugated to horseradish peroxidase and diluted 1:3,000 in PBS-T. After five additional washes, the immunocomplexes were developed using enhanced horseradish peroxidase-luminol chemiluminescence (ECL Western blotting detection reagents; Amersham) and detected after exposure to photographic film (Hyperfilm-ECL) for 5–30 s. Band intensity was quantitated by a laser scanning densitometer (Molecular Dynamics).

Electrophysiological assessment. A linear array of two stimulating and eight recording microelectrodes (50 μm in diameter positioned from 1.5 to 5 mm from the stimulation site) was used for electrophysiological studies as previously described (5). In brief, after equilibration of samples in a custom-built test chamber, an XYZ mechanical micropositioner (Narishige, JA) was used to gradually advance the microelectrode array toward either the top surface of the construct or the epicardial surface of the ventricle. Stimuli (1-ms monophasic pulses) were applied at a rate of 60 beats/min at an initial amplitude of 0.1 V, which was then increased until each stimulus was captured (i.e., followed by a tissue response) in all recording electrodes in the sample. The corresponding amplitude, defined as the excitation threshold, represented the lowest stimulus resulting in stable impulse propagation at 60 beats/min. The tissue was stimulated at 60 beats/min for 15 min, and the stimulation rate was then increased progressively. The maximum stimulation rate at which the sample could be captured for 5 min was defined as the maximum capture rate. Activation times at each recording electrode were determined as the minima of five-point derivatives of the low-pass-filtered signals. The average conduction velocity was calculated as the slope of the linear regression of activation time versus the interelectrode distance. The maximum amplitude was defined as the maximum value recorded from all electrodes at 60 beats/min (5). At the end of each experiment, tissues were simulated at frequencies higher than the maximum capture rate in an attempt to induce arrhythmias (5).

Statistics

Data were calculated as means ± SE and analyzed using one-way ANOVA followed by Fisher’s protected least-significance-difference post hoc test. Differences were considered statistically significant when P < 0.05. All calculations were performed using SuperANOVA III for Macintosh (SAS Institutes).

RESULTS

Cell Seeding

The use of sPGA instead of PGA scaffolds resulted in constructs with higher DNA contents after 1 h of seeding on the XYZ gyrator (443 ± 36 vs. 262 ± 33 ng DNA/mg wet wt, respectively, n = 17, P < 0.01). Cell attachment was significantly enhanced by seeding scaffolds with a concentrated cell suspension compared with inoculating HARVs with cells at lower initial concentration but at the same initial number of cells per scaffold. In particular, scaffolds seeded in grafted 96-well plates and flasks for 2 h, respectively, contained 466 ± 45 and 420 ± 68 ng DNA/mg wet wt, whereas scaffolds seeded in HARVs for 2 h contained less DNA, only 142 ± 24 ng DNA/mg wet wt (n = 16, P < 0.01).

Tissue Cultivation

The histological appearance of 1-wk constructs did not vary among the four experimental groups (sPGA/flask/high serum, sPGA/HARV/high serum, sPGA/HARV/low serum, and lsPGA/HARV/low serum). H-E staining showed a 120- to 160-μm-thick multicellular layer at the periphery of 1.3- to 1.4-mm-thick constructs in which myocytes were attached to one another and/or randomly oriented polymer fibers in a 3D configuration, as previously shown (5). Transmission electron microscopy demonstrated the presence of subcellular elements characteristic of cardiac myocytes, including myofilaments with well-defined sarcomeres that were 1.7–1.9 μm in length (Fig. 1A). Sarcomere length in the engineered cardiac muscle was thus similar to that previously reported for native rat ventricular tissue in the resting state (13) and to that we previously observed (5, 6). Immunohistochemical staining showed that the majority of cells (60 ± 4%, n = 6) at the construct periphery expressed the muscle-specific protein sarcomeric tropomyosin (Fig. 1B).

A thin, uniform coating of laminin was observed by immunofluorescence on lsPGA fibers both longitudinally (Fig. 2A) and in cross sections (results not shown) but not on uncoated sPGA fibers (Fig. 2A, inset). Esterase activity in the majority of cells located up to 100

Fig. 1. Muscle-specific features of engineered cardiac tissue [1-wk construct from the laminin-coated, surface-hydrolyzed polyglycolic acid (lsPGA)/high-aspect ratio vessel (HARV)/low-serum group]. A: transmission electron micrograph showing myofibrils (myf), sarcomeres with Z lines (z), abundant mitochondria (mit), and the extracellular space of two adjacent cells (arrows). Scale bar 500 nm. B: immunohistochemical staining for tropomyosin (brown color). *Undegraded polymer. Scale bar 15 μm.
mm deep to the construct surfaces demonstrated the viability of the peripheral tissue layer (Fig. 2B). Immunofluorescent microscopy also demonstrated that Cx43 distributions between adjacent cells appeared punctate and spatially uniform over the cell membrane in both engineered constructs and neonatal ventricles (Figs. 2, C–F). Cx43 staining at individual gap junctions was comparable for constructs and neonatal ventricles as assessed by fluorescence areas (2.00 ± 0.1 and 1.84 ± 0.21 pixels², n = 3) and intensities (88.8 ± 4.88 and 82.0 ± 9.16 normalized units, n = 3). In contrast to either constructs or neonatal ventricles, Cx43 staining of adult ventricles was confined to end-to-end cell connections (data not shown) as previously reported (42).

Electrophysiological studies showed that neither constructs nor neonatal ventricles exhibited any spontaneous beating, but both responded to electrical stimulation over a wide range of frequencies (up to 400 beats/min in constructs and 600 beats/min in ventricles; results not shown). Representative examples of impulse propagation in constructs and neonatal ventricles are shown in Fig. 3. The propagated waveforms had biphasic, smooth shapes, implying macroscopically continuous propagation without wave collisions. Conduction times increased linearly with distance over 5 mm (r² > 0.97), implying similar conduction velocities between adjacent electrodes and thus relatively homogeneous electrical properties in the peripheral cardiac tissue-like zone, as previously described (5). In response to electrical stimulation, constructs exhibited synchronous contractions that were visible to the unaided eye, indicating electromechanical coupling and a functional myofibrillar apparatus.

Constructs from the four groups were compared with each other and to neonatal ventricles with respect to biochemical indexes (cellularity, hypertrophy; Fig. 4, A and B), electrophysiological parameters (conduction velocity, maximum capture rate, maximal signal amplitude, and excitation threshold; Fig. 5, A–D), and molecular indexes (expression and phosphorylation of Cx43 and expression of CK-MM and MHC; Figs. 6 and 7). No significant cell proliferation occurred over 1 wk of cultivation on the basis of construct DNA content and medium LDH concentration, as previously reported (5).

Mixed flasks versus rotating bioreactors (i.e., the sPGA/flask/high-serum group versus the sPGA/HARV/high-serum group). The cumulative LDH release and molar lactate-to-glucose ratio were significantly higher (P < 0.01 and P < 0.05, respectively) in the sPGA/flask/high-serum group (17.7 ± 1.1 U/l and 1.3 ± 0.1, respectively) than sPGA/HARV/high-serum group (9.5 ± 1 U/l and 0.6 ± 0.1, respectively), indicating that rotat-
ing bioreactors supported the cultivation of a larger number of viable cells with more aerobic cell metabolism than mixed flasks. Lower cellularity (Fig. 4A) and a higher hypertrophy index (Fig. 4B) were observed in the sPGA/flask/high-serum group than sPGA/HARV/high-serum group (P < 0.05 and P < 0.01, respectively). Maximum capture rates (Fig. 5B) and the protein expression of Cx43 and CK-MM (Fig. 6, A and B, respectively) were all significantly lower (P < 0.05) in the sPGA/flask/high-serum group than sPGA/HARV/high-serum group. The incidence of tachyarrhythmia, induced by rapid stimulation (5), was significantly higher in the sPGA/flask/high-serum group than sPGA/HARV/high-serum group (5 of 10 and 0 of 10, respectively, P < 0.01).

Low versus high serum concentration (i.e., the sPGA/HARV/low-serum group versus the sPGA/HARV/high-serum group). No significant difference in biochemical indexes was observed between the two groups (Fig. 4). The maximum signal amplitude (Fig. 5C) and protein expression of MHC (Fig. 6C) were significantly higher (P < 0.05) in the lsPGA/HARV/low-serum group than sPGA/HARV/low-serum group.

Laminin versus no laminin coating of polymer scaffolds (i.e., the lsPGA/HARV/low-serum group versus sPGA/HARV/low-serum group). The hypertrophy index (Fig. 4B), a measure of cell size, was higher for the laminin-coated than uncoated scaffolds, whereas in a previous report laminin-coated substrates increased the surface area of cardiac myocytes (20). The conduction velocity (Fig. 5A) and expression level of CK-MM (Fig. 6B) were significantly higher (P < 0.01) in the lsPGA/HARV/low-serum group than sPGA/HARV/low-serum group.

Neonatal ventricles versus 1-wk constructs. The cellularity of constructs from the lsPGA/HARV/low-serum group was 25% of that in neonatal ventricles (Fig. 4A; P < 0.001), which was 51% higher than in our previous
study (5). Constructs and ventricles had similar cell hypertrophy indexes (Fig. 4B), as previously reported (5, 6). Conduction velocities measured for native rat tissues were consistent with previously published values (37, 38). Conduction velocities measured for constructs in the IsPGA/HARV/low-serum group were 87%
as high as those measured for ventricles (Fig. 5A), whereas in our previous study (5) construct conduction velocities were only 56 and 38% as high as those measured for neonatal and adult ventricles, respectively. Maximum capture rates in the lsPGA/HARV/low-serum group were 65% as high as those in neonatal ventricles (Fig. 5B; \( P < 0.03 \)) and 71% higher than in our previous study (5). Maximum signal amplitudes of constructs from the lsPGA/HARV/low-serum group were 12% as high as in neonatal ventricles (Fig. 5C, \( P < 0.0001 \)) and fourfold higher than in our previous study (5). Excitation thresholds were 2.5 times higher than in neonatal ventricles (Fig. 5D; \( P < 0.01 \)) and 42% lower than in our previous study (5). The incidence of tachyarrhythmia in the lsPGA/HARV/low-serum group was similar to that in neonatal ventricles.

Protein expression levels of Cx43, CK-MM, and MHC in the lsPGA/HARV/low-serum group were, respectively, 50, 50, and 40% as high as those measured in neonatal ventricles (Fig. 6, A, B, and C, respectively; \( P < 0.01 \)). A linear correlation was observed between the maximum capture rate (Fig. 5B) and protein expression of Cx43 (Fig. 6A; \( r^2 = 0.99 \)), which is consistent with previous reports that the maximum capture rate at which conduction block occurred depended on electrical coupling (24, 35). In constructs and neonatal ventricles, the 43-kDa Cx43 band collapsed into a 41-kDa band after treatment with alkaline phosphatase, suggesting that the majority of Cx43 in engineered tissues and neonatal ventricles was phosphorylated (i.e., in a functional state) (Fig. 7A) (27).

**DISCUSSION**

The present study demonstrates that the molecular, structural, and electrophysiological properties of engi-
neered cardiac muscle are interrelated, dependent on model system parameters, and can be improved by using a high concentration of cardiac myocytes, laminin-coated surface-hydrolyzed PGA scaffolds, rotating bioreactors, and low-serum medium. Compared with our previous study (5) in which constructs were grown using uncoated PGA scaffolds, mixed flasks, and high-serum medium, the best constructs in the present study (the lsPGA/HARV/low-serum group) had a 51% higher cellularity, twofold higher conduction velocity, fourfold higher maximum signal amplitude, 70% higher maximal capture rate, and 42% lower excitation threshold. Compared with native neonatal ventricles, the best engineered constructs had comparable conduction velocities and spatial distributions of Cx43 and expressed approximately one-half as much Cx43, CK-MM, and MHC.

The combined use of a highly concentrated cell suspension and surface-hydrolyzed PGA significantly improved the attachment of cardiac myocytes, as previously reported for vascular smooth muscle cells (17). In particular, the use of sPGA instead of PGA doubled the number of cells attached after 1 h, and the use of a high rather than low cell concentration during seeding tripled construct cellularity at 2 h. Surface modification of PGA scaffold by short-term exposure to strong alkaline conditions was previously shown to significantly increase the wettability and hydrophilicity of the PGA fibers while leaving unchanged the overall mesh structure, fiber diameter, and polymer molecular weight (17). Increased wettability of sPGA facilitated the process of loading a small volume of highly concentrated cells as well as the process of coating the mesh with a uniform layer of laminin (Fig. 2A), because proteins adsorb to hydrophilic surfaces at significantly higher rates than hydrophobic ones (3).

We previously showed that the thickness of the cardiac tissue-like zone at the construct periphery could be increased by seeding scaffolds with a cell population enriched with respect to cardiac myocytes (5) and that final construct cellularity could be improved by increasing the initial cell density and culturing under mixed conditions (6). In the present study, constructs from all four experimental groups (seeded with a high number of myocyte-enriched cells and cultured for 1 wk under mixed conditions) contained a 120- to 160-number of myocyte-enriched cells and cultured for 1 wk from all four experimental groups (seeded with a high concentration of cardiac myocytes, laminin-coated surface-hydrolyzed PGA scaffolds, rotating bioreactors, and low-serum medium. Compared with our previous study (5) in which constructs were grown using uncoated PGA scaffolds, mixed flasks, and high-serum medium, the best constructs in the present study (the lsPGA/HARV/low-serum group) had a 51% higher cellularity, twofold higher conduction velocity, fourfold higher maximum signal amplitude, 70% higher maximal capture rate, and 42% lower excitation threshold. Compared with native neonatal ventricles, the best engineered constructs had comparable conduction velocities and spatial distributions of Cx43 and expressed approximately one-half as much Cx43, CK-MM, and MHC.

The combined use of a highly concentrated cell suspension and surface-hydrolyzed PGA significantly improved the attachment of cardiac myocytes, as previously reported for vascular smooth muscle cells (17). In particular, the use of sPGA instead of PGA doubled the number of cells attached after 1 h, and the use of a high rather than low cell concentration during seeding tripled construct cellularity at 2 h. Surface modification of PGA scaffold by short-term exposure to strong alkaline conditions was previously shown to significantly increase the wettability and hydrophilicity of the PGA fibers while leaving unchanged the overall mesh structure, fiber diameter, and polymer molecular weight (17). Increased wettability of sPGA facilitated the process of loading a small volume of highly concentrated cells as well as the process of coating the mesh with a uniform layer of laminin (Fig. 2A), because proteins adsorb to hydrophilic surfaces at significantly higher rates than hydrophobic ones (3).

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Cx43 immunostaining (Fig. 2C and D) and protein expression by Western blot analysis (Figs. 6A and 7A) demonstrated the presence of newly formed gap junctions in the peripheral region of 1-wk constructs from all four groups. Cardiac gap junctions are membrane specializations that permit the passage of ions and small signaling molecules between myocytes, thus facilitating electrical conduction and chemical communication (31). The size and spatial distribution of myocyte gap junctions changes during development from a punctate distribution over the entire cell membrane in neonates to a confined distribution at end-to-end cell connections in adults (21, 43). The finding that Cx43 was phosphorylated (the 43-kDa band collapsed into a 41-kDa band after alkaline phosphatase treatment) (Fig. 7A) implies that the gap junctions that formed during construct cultivation were functional (27).

The protein expression levels of CK-MM and MHC in constructs were quantified as markers of cellular differentiation. Creatine kinase, a dimer of M- or B-type subunits, plays a vital role in the maintenance of cytosolic ATP (29), whereas MHC is involved in the generation of contractile force (29). During cardiac tissue development and differentiation, the muscle-specific CK-MM isoenzyme (22, 40) and the contractile protein MHC (18) are significantly upregulated.

Rotating bioreactors improved construct cellularity (DNA content; Fig. 4A), cell survival and metabolism (medium LDH activity and lactate-to-glucose ratio), maximum capture rate (Fig. 5B), and expression of muscle-specific molecular markers Cx43 and CK-MM (Fig. 6A and B). In mixed flasks, lower expression of Cx43, indicative of decreased intercellular coupling, was associated with higher construct arrhythmogenicity, which is consistent with previous reports (21, 32). These findings suggest that rotating bioreactors improved the viability, electrical coupling, and differentiated phenotype of cultured cardiac myocytes. We previously attributed similar results obtained for both engineered cartilage (14, 15, 45) and cardiac muscle (6) to the dynamic laminar flow conditions present in the rotating vessels compared with the turbulent conditions present in mixed flasks. A higher degree of cellular differentiation in rotating bioreactors has also been observed for a variety of cell types, including renal and hepatic cells (reviewed in Ref. 41). The finding that the use of rotating bioreactors rather than mixed flasks improved the properties of constructs suggests that further bioreactor design efforts should be aimed at providing mass transport to cultured tissues without introducing mechanical signals (e.g., turbulent shear) that can cause cell damage or dedifferentiation.

High-serum medium was used for the first 2 days of cultivation in an attempt to maximize initial cell attachment and support the limited mitogenic activity of neonatal cardiac myocytes (40). Two groups (sPGA/HARV/low serum and lsPGA/HARV/low serum) were then switched to low-serum media in an attempt to promote cardiac myocyte differentiation (25, 33, 40) and attenuate proliferation of nonmyocytic cells (30). In contrast, high-serum medium was shown to promote cardiac myocyte dedifferentiation by inhibiting the expression of CK-MM, reducing the stability of MHC and cardiac α-actin expression (9, 19, 28, 33). Constructs from the low-serum groups upregulated MHC expression (Fig. 6C) compared with those from the high-serum groups, suggesting that low serum promoted differentiation of the contractile apparatus in 3D cul-
tures of cardiac myocytes, as was previously reported for monolayer cultures (25).

In monolayer cultures, laminin coating has been shown to improve cardiac myocyte attachment and spreading (4) and induce myofibrillogenesis (10, 20) through association with specific integrin receptors. In the present study, the use of laminin-coated 3D substrates (the lSpGA/HARV/low-serum group) resulted in constructs with the highest conduction velocities (Fig. 5A), which could be attributed to changes in intercellular coupling, electrical membrane properties, size, shape, and/or packing density of cardiac myocytes (36). Laminin coating of polymer fibers possibly affected construct conduction velocity by promoting increased cell size (Fig. 4B), because no change in intercellular coupling, cell shape, or cell packing density could be attributed specifically to the presence of laminin. It is also possible that laminin coating affected conduction velocity by changing the electrical membrane properties of the cardiac myocytes in a manner previously reported for neurons (2). In addition, the use of laminin-coated substrates significantly increased construct amounts of CK-MM (Fig. 6A), suggesting that laminin promoted cardiac myocyte differentiation, as previously described (4, 10, 20).

In the present study and our previous study (5), engineered cardiac tissue was functionally evaluated with respect to four parameters (conduction velocity, excitation threshold, maximum capture rate, and signal amplitude), which, although dependent on construct cell density and intercellular coupling, allowed us to compare constructs cultured under different conditions and are expected to help in the development of improved methods of culture. Engineered constructs in the lSpGA/HARV/low-serum group had conduction velocities comparable to those of native ventricles (Fig. 5A) but expressed only 60% as much Cx43 (Fig. 6A), which is consistent with a recent report (24) that a noticeable decrease in conduction velocity requires a major decrease in intercellular coupling. However, the lower expression levels of Cx43 and lower cellularities (Fig. 4A) of constructs can account for their inferior maximum capture rates, maximum amplitudes, and excitation thresholds compared with neonatal ventricles (Fig. 5, B, C, and D, respectively) (5).

The 3D culture system used in the present study was superior to those used previously (5, 6), permitted cardiac myocytes to express their differentiated phenotype, and provided a valuable in vitro model to study the development and function of cardiac muscle. The potential clinical usefulness of engineered cardiac muscle will require significant improvements in its electromechanical properties and the reconstruction of the functional heterogeneity present in vivo. In ongoing work, we are attempting to further characterize the engineered cardiac muscle by cell-level electrophysiological studies and gene-level differentiation analyses and to develop tissue culture bioreactors that better mimic the in vivo physiological environment.

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