Controlling the Cellular Organization of Tissue-Engineered Cardiac Constructs

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ABSTRACT: There are currently no effective treatments to restore the cardiac muscle lost because of ischemia for the millions of people who suffer heart attacks annually. Cell therapy procedures have emerged as novel therapeutic strategies for treatment of heart failure after myocardial infarction but have been hampered by the lack of adequate cell sources of cardiomyocytes and by the inability to integrate cell grafts into cardiac muscle. A cardiac patch composed of organized and functional cardiomyocytes could drastically enhance the efficacy of this important clinical approach. Here, we report our ongoing efforts to develop a bioartificial cardiac muscle capable of synchronized multidirectional contraction within a three-dimensional hydrogel scaffold. Neonatal rat cardiomyocytes, smooth muscle cells, and reconstituted polymeric collagen enriched with growth factors and hormones are used. A bioreactor system is used to impart precise strains onto the developing tissue constructs *in vitro*. The results demonstrate that cell-mediated collagen compaction is significantly enhanced by strain preconditioning, resulting in a more favorable cellular organization. Furthermore, the results demonstrate that strain stimulation guides cellular orientation in the direction of applied strain (i.e., in the circumferential direction). Hence, we demonstrate the importance of mechanical preconditioning as a means of promoting the *in vitro* development of engineered cardiac muscle for use with myocardial regeneration therapies.

KEYWORDS: cardiomyocytes; collagen hydrogel; bioreactor; mechanical stimulation; *in vitro* development

INTRODUCTION

The adult heart has a limited capacity to regenerate itself, and damage to the heart muscle usually results in irreversible cardiac dysfunction. The most common form of cardiac dysfunction occurs as a result of ischemic heart disease and myocardial infarction (MI). MI leads to a significant loss of cellular viability and diminished cardiac performance. Prolonged cardiac dysfunction ultimately leads to the development of progressive heart failure. In the United States alone, heart failure affects more than 5 million people, with 400,000 new cases reported each year.[1] The con-

sequences of MI-related heart failure, mainly disability and mortality, place an enormous burden on health care systems and the economy.[1] Even with the recent technological advances of therapies for heart failure, the prognosis remains poor, and this epidemic continues to grow. With a limited supply of donors for whole-organ transplantation, the development of new therapies to deal with this epidemic has become imperative.

A possible novel approach for the treatment of heart failure may be the development of myocardial regeneration strategies aiming to replace part of the dysfunctional myocardium with new contractile tissue.[2] Several myogenic sources have been suggested as potential sources for tissue grafting, including skeletal myoblasts,[3,4] fetal cardiomyocytes,[5,6] smooth muscle cells,[7–9] embryonic stem cells, and bone marrow–derived stromal and hematopoietic stem cells.[10,11] Recent animal studies have shown that cells derived from all sources may survive up to a certain degree, differentiate within the host myocardium, and even improve cardiac function.[4,10] Although several myocyte preparations have been used in these studies, the inherent electrophysiological, structural, and contractile properties of cardiomyocytes strongly suggest that they may be the ideal donor cell type. Cardiomyocytic cell transplantation in small animal models of myocardial infarction or cryoinjury was associated with smaller infarcts, prevented cardiac dilatation and remodeling after myocardial infarction, and improved the ventricular function in some of these studies.[12–14]

Despite these initial encouraging results, several considerable obstacles must be surmounted before this approach can gain acceptance on a clinically relevant scale. The critical issue is the lack of adequate cell sources for human cardiomyocytes for cell transplantation. Adult cardiomyocytes are terminally differentiated, and human fetal or neonatal cardiomyocytes cannot be obtained in sufficient numbers, because of practical and ethical reasons. Another important issue is the functional donor-to-host integration. Despite the initial reports showing the feasibility of engrafting several different types of myocytes, it is still not established whether these cells actually can integrate functionally with the preexisting myocardial tissue. Optimal functional improvement would require structural, electrophysiological, and mechanical coupling of donor and host tissue. Current cell therapy paradigms are based on delivering the cells to the injury site by injecting a bolus of cellular suspension into the subinterstitial space of the ischemic cardiac muscle. This strategy, however, suffers from several drawbacks. (1) Only a minority of the cells (<10%) actually survive after cell transplantation. (2) The thickness of the new muscle that can be formed is rather limited and therefore may have a limited impact on the mechanical properties of the failing heart. (3) The graft properties—namely, the number and density of cells within the graft and the cells' orientation and electromechanical connections—cannot be controlled. (4) The inadequacy of graft vascularity limits cell survival and graft size. New advances in tissue engineering technology may open up new opportunities for myocardial regeneration procedures and provide innovative solutions for some of the most critical obstacles.

Our ongoing research efforts are focused on the development of an engineered cardiac construct (ECC) comprising cardiomyocytes, smooth muscle cells, and type I collagen for use as a contractile myocardial patch. The coculture ECCs were developed using collagen gels prepared with appropriate growth factors to provide the cardiac cells with the correct three-dimensional environment and ample biomechanical and biochemical support. This approach proved capable of providing control over

graft shape and size, while at the same time promoting cell survival and tissue function. To improve the structural characteristics of the graft tissue, we have preconditioned the ECCs with mechanical stimulation in the form of an externally applied cyclic distension. The distensions were transmitted to the developing tissue in a bioreactor system capable of mimicking the natural myocardial wall motion *in vitro*. ECCs exposed to several days of preconditioning exhibited a substantial reorganization in tissue structure and cellular orientation. The cell orientation in preconditioned constructs was orthotropic, which is more favorable for donor–host tissue coupling. Hence, *in vitro* mechanical stimulation and cell preconditioning may prove of vital importance in cell therapies aimed at developing new contractile tissue for restoring physiological performance to dysfunctional myocardium.

METHODS

Cell and Tissue Culture

Proliferating sheep aortic smooth muscle cells (SMCs) were isolated from fresh aortic explants and maintained on tissue culture polystyrene at 37°C and 5% CO_2 using standard tissue culture protocols. Cells were sustained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK) with 10% fetal bovine serum (Gibco), 1% L-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel), and 1% penicillin-streptomycin (Biological Industries). SMCs (between passage 4 and passage 8) were used to create cardiac constructs. Neonatal rat cardiomyocytes were freshly harvested from 2–3-day-old rat pups according to published protocols.[15,16] The cardiomyoctyes were cultured in DMEM with 10% fetal calf serum (Gibco), 1% L-glutamine (Biological Industries), and 1% penicillin-streptomycin (Biological Industries).

Engineered Cardiac Constructs

ECCs, composed of a coculture of aortic smooth muscle cells and cardiac myocytes, were prepared according to the modified methods of Zimmermann *et al.*[17,18] In brief, a precursor solution (12 mL) composed of purified rat-tail type I collagen (4 mg/mL; 1.92 mL), basement membrane extract (1.2 mL; R&D Systems, Minneapolis, MN), 0.1 N NaOH (800 µL), $2 \times$ DMEM (1.92 mL), horse serum (2.4 mL; Biological Industries), chick embryo extract (480 µL), penicillin-streptomycin (240 µL), 2×10^7 SMCs (1.5 mL), and 1×10^7 neonatal cardiomyocytes (1.5 mL) was mixed and cast into test tubes containing a 10N sulfuric acid–etched silicone support bulb. The precursor solution underwent a spontaneous polymerization process to form a highly entangled network of collagen fibrils, thereby physically entrapping the coculture of cells. Subsequent cell-mediated compaction of the ECCs resulted in the densification of the collagen to form a thin myocardial-like tissue, which was firmly anchored onto the acid-etched silicone bulb.

Mechanical Stimulation Bioreactor

The cell-seeded construct and underlying silicone bulb were transferred into a sterile mechanical stimulation bioreactor 2 days after the initial ECC preparation
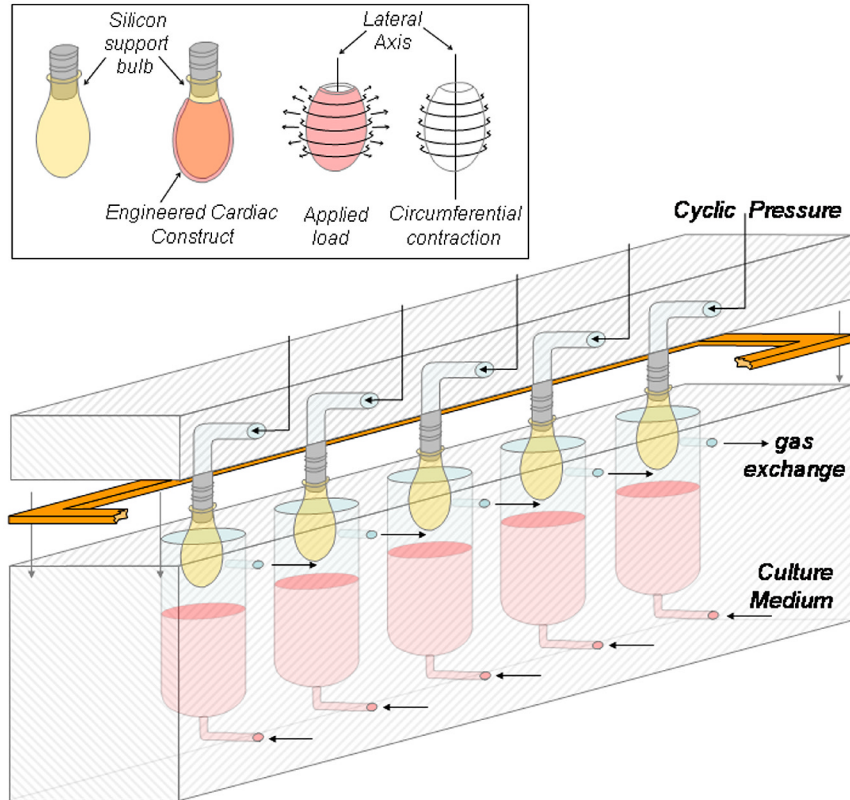
FIGURE 1. Strain stimulation bioreactor for the *in vitro* cultivation of engineered cardiac constructs (ECCs). Five ECCs are cultured simultaneously in separate chambers on top of silicone support bulbs. Cyclic pressure inflates the silicone bulbs, causing applied load in the circumferential and radial directions **(top panel)**.

(FIG. 1). The culture medium in the bioreactor contained DMEM with 10% horse serum (Biological Industries), 2% chick embryo extract, and 1% penicillin-streptomycin (Biological Industries). The bioreactor was designed to impart exact cyclic strain to the ECCs by inflating the underlying silicone bulb with repetitive pneumatic pressure. The amplitude, frequency, and duty cycle of cyclic distension was prescribed by pneumatic control with electric solenoid valves, pressure regulators, and digital control circuitry. In this study, duty cycle is defined as the on-time and off-time of the pressurization of the silicone bulb during a 1-s interval (e.g., 0.7/0.3 represents a 0.7-s pressurization and 0.3-s depressurization). Calibrating the bioreactor system and control module was performed before experimentation. The experimental protocol for mechanical stimulation involved placing the ECC-mounted silicone bulb into the medium-filled bioreactor where it was connected to an inlet port. A Lure-lock-connector (Value Plastics, Fort Collins, CO) ensured a watertight seal between the silicone bulb and the outside. With the bulb in place, the chamber was sealed, and
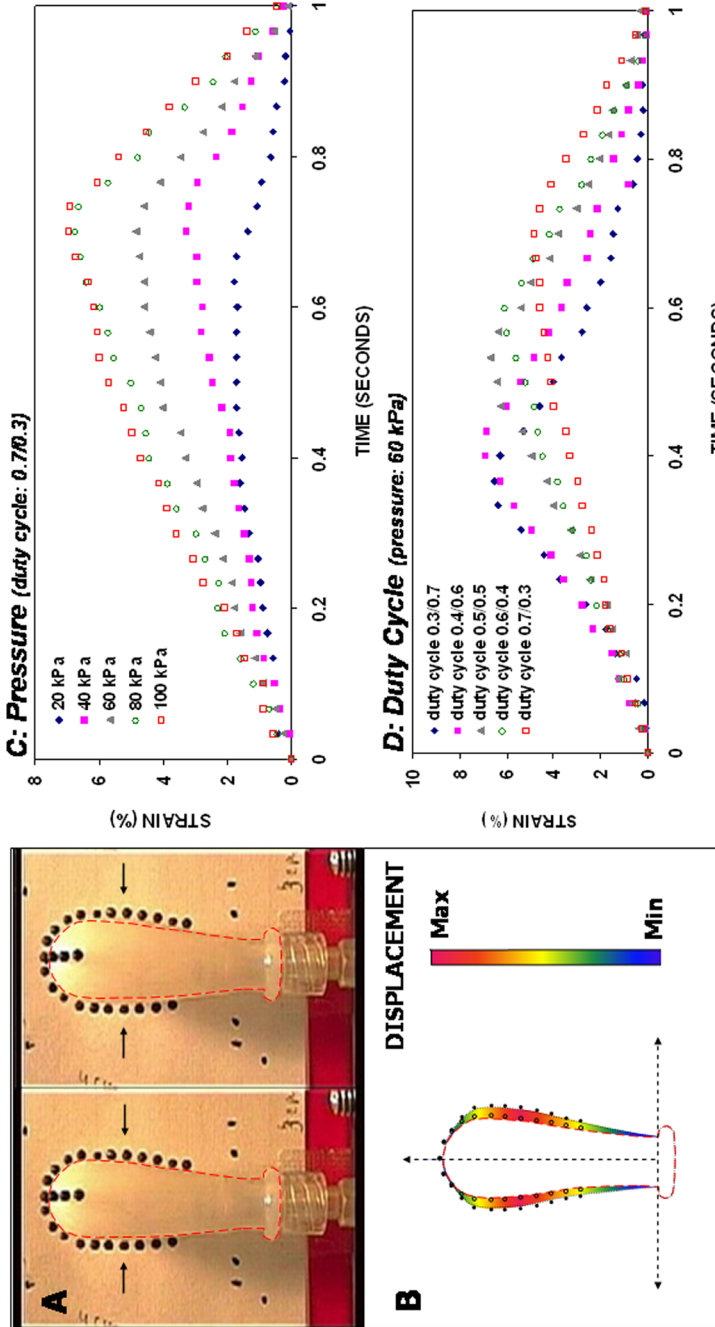
FIGURE 2. Bioreactor calibration. Silicone bulbs are outfitted with small beads on the outer periphery. Bead location is tracked with image analysis software, and strain measurements are calculated accordingly. **(A)** *Arrows* on the images indicate the point of maximum strain. **(B)** Strain distribution on the silicone bulb is determined from the bead displacement. **(C)** The strain-versus-time profiles are plotted for different inflation pressures and **(D)** duty cycles.

0.2-μm filters were placed in the vents to allow for gas exchange. Finally, the entire apparatus was placed inside a CO_2-regulated incubator kept at 37°C and connected to a regulated compressed air supply through a solenoid valve that vented to atmosphere. The intraluminal pressure to which the bulbs were exposed was regulated to produce a 0–12% cyclic change in the outer diameter of each silicone bulb (FIG. 2). Mechanical preconditioning involved repeated inflation and deflation of the bulbs by this amount at a 1-Hz frequency for periods of 6 days. Each dynamic test was accompanied by a control experiment in which an equal number of bulb/constructs were cultured in the incubator for an equivalent amount of time but under static conditions. At the conclusion of each experiment, the experimental and control ECCs were removed and the constructs detached from the outer surface of the silicone bulb.

Strain and Strain Rate Calibration

The calibration procedure involved placing small black beads on the perimeter of the silicone bulb and monitoring these beads with a high-resolution charge-coupled device camera at 60 frames per second (FIG. 2). The beads were digitally tracked with Scion Image for Windows (Scion Corporation, Frederick, MD), and their position was documented in each frame during the entire 1-s duty cycle. Stationary beads placed in the background also were tracked and used as a fixed reference frame during the displacement measurements. The individual bead displacement from frame to frame was converted to strain through normalization by the initial diameter of the unpressurized silicone bulb. This analysis yielded the two-dimensional strain distribution along the perimeter of the silicone bulb during the course of the duty cycle. Strain rate was determined by plotting the bead strain as a function of time for each individual bead (FIG.2). The strain was characterized at five inflation pressure set points (i.e., 20, 40, 60, 80, 100 kPa) and with five different duty cycles (0.3/0.7, 0.4/0.6, 0.5/0.5, 0.6/0.4, and 0.7/0.3).

Assessment of Cellular Organization

Cellular organization was assessed by cryosectioning and cytohistochemistry techniques.[19] At the conclusion of each experiment, the ECCs were fixed in 2% paraformaldehyde for 30 min, and samples of tissue from each construct were placed in OCT freezing medium (Sakura, CA) and flash-frozen in liquid nitrogen–cooled 2-methylbutane. The frozen samples then were mounted on metal chucks and sectioned in orthogonal planes (5–12 μm thick), perpendicular to and parallel to the axis of the silicone bulb. Representative sections were stained with hematoxylin and eosin (H&E) (Sigma Diagnostic, St. Louis, MO), showing cell nuclei in blue (hematoxylin) and extracellular matrix in pink (eosin-Y). Collagen fibril alignment and organization were assessed on unstained cross-sections of tissue sample. Thin sections were placed on glass slides and allowed to dry for 30 min at room temperature. The slides were then fixed for 5 min in acetone (−20°C) and then allowed to air dry for 15 min at room temperature. Dried sections were rehydrated in phosphate-buffered saline for 5 min at room temperature, mounted with cover slips using Flouromount G (Southern Biotechnology Associates, Birmingham, AL) and examined by phase-contrast microscopy.

TABLE 1. Maximum strains and strain rates

|	Duty cycle				
	0.3/0.7	0.4/0.6	0.5/0.5	0.6/0.4	0.7/0.3
Strain (percent)					
20 kPa	1.6	1.8	1.5	1.5	1.8
40 kPa	4.2	3.9	4.2	3.5	3.3
60 kPa	6.5	6.9	6.7	6.1	4.8
80 kPa	–	10.8	9.2	8.2	6.7
100 kPa	–	–	11.1	9.8	6.9
Strain rate (seconds^{-1})					
20 kPa	2.2	4.1	2.8	3.1	3.5
40 kPa	11.1	8.2	7.9	6.1	4.8
60 kPa	16.4	16.2	12.1	10.4	7.7
80 kPa	–	23.8	16.9	13.5	10.1
100 kPa	–	–	21.5	17.2	10.7

RESULTS

Bioreactor Calibration

The strains transmitted to the ECCs by the bioreactor system were well characterized before experimentation. Specifically, the calibration of the bioreactor system involved characterizing strain distribution on the silicone bulb as a function of inflation pressure and duty cycle. Strain distribution was determined to be axisymmetric along the lateral axis of the bulb based on measurements made on one side and compared with those of the opposite side (data not shown). Maximum strains were always observed on the bulb in the region located 2/3 up from the base (FIG. 2A, indicated by arrows). The strain was distributed along the longitudinal wall of the bulb, and no strain was measured on the top-most portion of the bulb (the cap). The strain rate was constant during inflation and exhibited nonlinear properties (with respect to time) during the deflation portion of the duty cycle. A linear relationship between inflation pressure and strain was observed only at the lower inflation pressures (<60 kPa). The relationship between strain rate and duty cycle proved to be nonlinear, too. The maximum rate of strain of the system (23.8/s) was recorded with an inflation pressure of 80 kPa and a 0.4/0.6 duty cycle. Likewise, maximum strain (11.1%) was recorded at an inflation pressure of 100 kPa and a 0.5/0.5 duty cycle. TABLE 1 summarizes the maximum strains and strain rates at all the inflation pressures and duty cycles tested.

ECC Compaction

ECCs were molded over a silicone bulb and allowed to statically compact and adhere to the acid-etched silicone surface for two days. ECCs were routinely checked to ensure that they were firmly adherent to the silicone surface before preconditioning in the bioreactor. During the initial two-day compaction, SMCs facilitated most
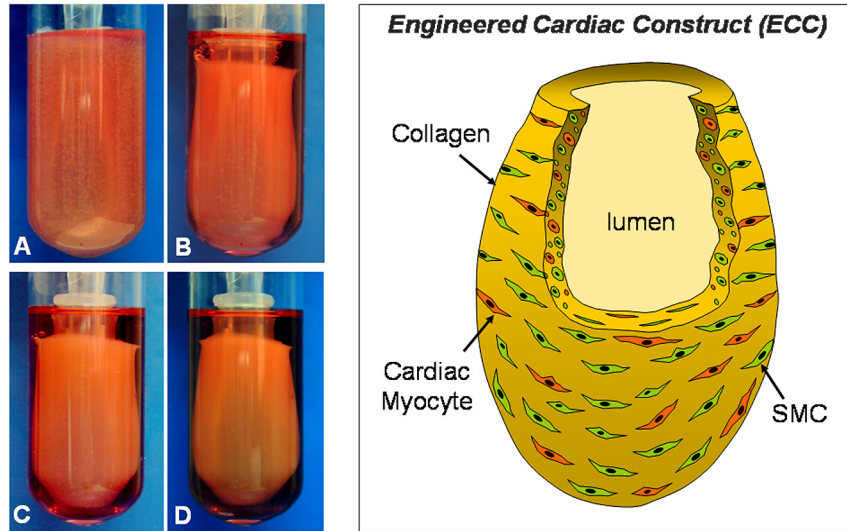
FIGURE 3. Engineered cardiac constructs (ECCs). A reconstituted type I collagen solution containing smooth muscle cells and cardiomyocytes was polymerized and compacted on top of a silicone bulb. The gross macroscopic appearance of the ECCs was recorded at time = 0, 6, 12, and 24 h after gel casting (**A–D**, respectively). **Right panel:** Schematic illustration of the cellular composition of the ECC after compaction.

of the compaction; ECCs comprised of neonatal cardiomyocytes alone were significantly less compacted as compared with ECCs made with SMCs (data not shown). Control constructs made without cells (acellular controls) polymerized into hydrogels but did not compact thereafter. FIGURE 3A–D demonstrates the process of compaction and adherence of an ECC to the silicone bulb over the course of 48 h. As the construct compacted, the collagen gel became denser, and the fluid was displaced from within the interstices of the hydrogel network. As a result, the collagen fibrils that comprised the construct reoriented drastically over the initial 48-h compaction. Phase contrast micrographs of the collagen fibrils demonstrated an isotropic orientation throughout the ECC at the initial phase of compaction and anisotropic orientation after gel compaction.

Cellular and Structural Organization

Cell distribution was assessed in each construct immediately after gel casting and after several days of static and dynamic incubation. The distribution of cells was found to be homogeneous throughout the constructs, and homogeneity was not influenced by the application of strain stimulation. The orientation of fibrils in ECCs exposed to mechanical preconditioning depended on their location within the construct (FIG. 4). Fibrils were organized along the circumferential axis of the construct throughout the midportion of the ECC, where the strain was highest. Near the upper region of the construct (i.e., the cap) the fibrils were mostly randomly oriented. In

the regions of highest strain, collagen fibrils formed organized clusters in the direction of the applied load (i.e., circumferentially). In contrast, collagen fibrils near the cap of the construct did not form any organized structures, and less-organized collagen fibril clusters were observed near the neck of the construct (FIG. 4A-A). The orientation of the cells inside the ECCs was highly correlated with the organization
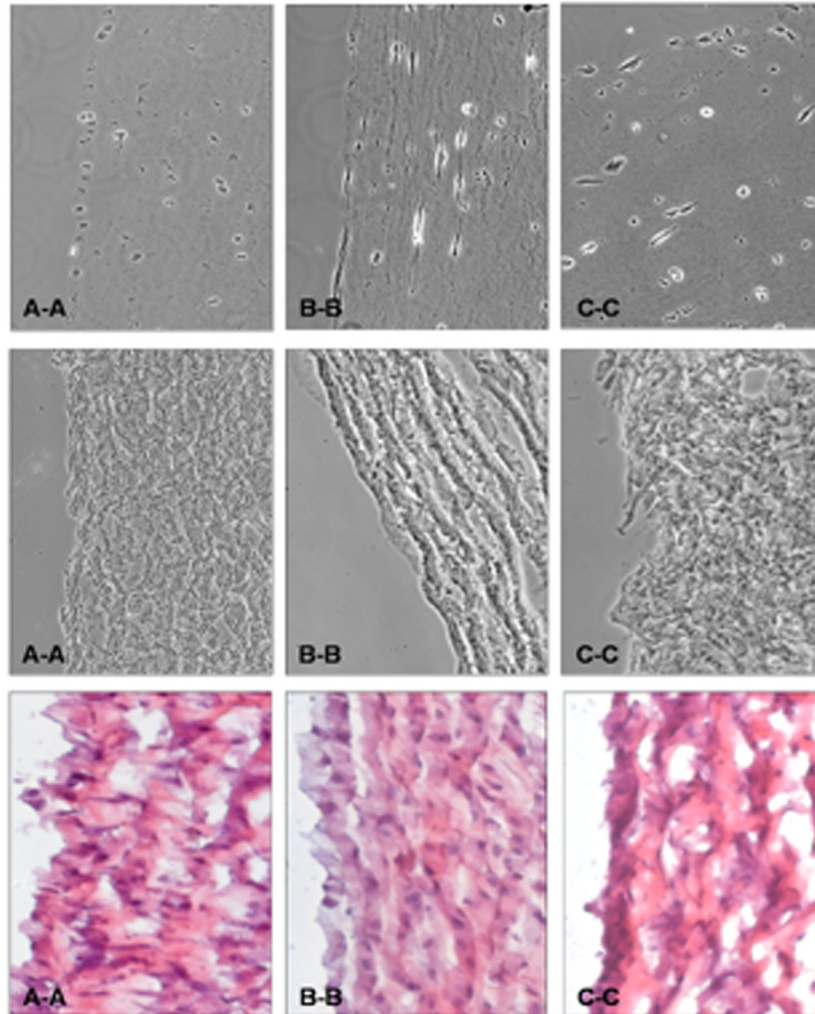
FIGURE 4. Cellular organization of mechanically preconditioned engineered cardiac constructs. Histological cross-sections of ECCs examine the cellular organization and collagen fibril orientation in the constructs after being exposed to strain stimulation for up to 6 days. Sections are taken from **(A-A)** the neck of the construct, **(B-B)** the midsection, where strain is maximal, and **(C-C)** the cap. Cellular organization **(top panels)** and fibril organization **(middle panels)** are influenced by the degree of strain, as determined by the location on the ECC.

of the collagen fibrils. Cellular orientation in the neck of the construct was characterized by bands of oriented cells near the inner wall with randomly oriented cells elsewhere. In contrast, most cells near the cap of the construct were randomly oriented, whereas cells in the midsection of the construct were circumferentially oriented throughout the thickness of the wall. Static control constructs that were incubated without strain stimulation exhibited randomly oriented cells and collagen fibrils throughout the construct (data not shown). H&E micrographs of ECC cross-sections (FIG. 4, bottom) show a high number of cell nuclei (dark) surrounded by dense collagen and extracellular tissue (light) in all locations of the ECC.

DISCUSSION

The development of myocardial regeneration strategies is important because of the overwhelming patient need and the limitations of standard replacement therapies. Cardiac cell transplantation recently has emerged as the leading strategy for replacing damaged or diseased myocardium.[2] Nevertheless, cell therapies are limited by the inability to locally sustain large transplanted cell grafts in the host tissue.[2] Furthermore, it is not evident that cell injections alone, without an organized structural support, will, indeed, functionally engraft into the host myocardium after implantation.[2] A tissue engineering approach for generating viable and contractile myocardial tissue ultimately could be used to augment the function of the diseased heart.

Our strategy utilizes a collagen scaffold, which supports a coculture of contractile SMCs and neonatal cardiomycytes. Unlike other studies that use a mixed population of neonatal cardiac cells, our approach takes advantage of the unique ability of the SMCs to efficiently compact the collagen scaffold and thus bring the entrapped cells into close proximity with one another.[20] This is particularly important in the context of a myocardial tissue substitutes, in which cell-to-cell contact is crucial for contractile function.[21] We observed that the use of SMCs in the constructs resulted in significant compaction of the collagen gels and thus led to a myocardial tissue substitute containing a high density of cells (FIG. 4). We also observed that ECCs made of neonatal cardiomyocytes alone were considerably less compacted. The SMC-mediated compaction of the collagen scaffold thus improved the architectural features of the ECCs, including the proximity of cells to one another. Consequently, cell proximity is thought to be a key aspect of the structure–function relationship of native myocardial tissue.[2]

Cell-mediated restructuring and reorganization of the collagen scaffold was promoted by using dynamic mechanical stimulation.[20,22] This strategy involved the application of cyclic strain onto the cell-seeded ECCs, by using a special bioreactor system developed for this purpose. The bioreactor, illustrated in FIGURE 1, was designed to simultaneously apply dynamic stimulation onto five independent ECCs under sterile conditions. The pneumatic application of strain was precisely controlled to allow for a range of loading pressures and duty cycles that render different strains and strain rates onto the ECCs. A comprehensive calibration of the system was performed to correlate loading pressure and duty cycle to the distensions of the outer wall of the silicone bulb. Consequently, we were able to determine the exact strains and strain rates experienced by the ECCs at any time point in the loading cycle. Fur-

thermore, this information allowed one to prescribe a contrived or physiological strain regimen by controlling both the duty cycle parameters (i.e., on and off times) and the inflation pressure of the system.

In the process of calibrating the bioreactor system, we discovered that the distribution of dynamic strain on the silicone bulb was not uniform. The uppermost region of the silicone bulb (i.e., the cap) exhibited negligible strains, whereas the magnitude of strain below the cap region increased along the length of the bulb and became maximal 2/3 down the length of the bulb, followed by decreasing strain thereafter (FIG. 2B).

Dynamic Strain and Cellular Organization

The orientation of cardiomyocytes in the heart has a profound impact on the contractile function of the heart muscle. Hence, our efforts to engineer a cellularized cardiac tissue substitute must take into account cellular organization and consider how to control this cellular organization. We hypothesized that strain stimulation could be used as a method of preconditioning the ECCs and thus causing reorganization within the tissue to prescribe a specific cellular orientation.[18] The nonuniform distribution of strain along the length of the silicone bulb poses an interesting scenario for assessing the correlation between the magnitude of strain and cellular organization. The bioreactor provides the means of applying dynamic strain in varying degrees onto the same construct, and by measuring the resulting cellular reorganization at different locations in the construct we can correlate strain and local reorganization. Thus, the bioreactor can provide a wide range of strains and strain rates to further test our working hypothesis and to characterize tissue remodeling as a function of various parameters. In this study, we measured cellular reorganization in response to one specific loading condition: 6.7% maximum strain and 12.1/s maximum strain rate. We characterized cellular reorganization for both cell and collagen fibril organization, the latter being the consequence of cell-mediated remodeling events.[23,24]

Tissue samples were taken from three different locations of the ECCs and histologically assessed using light-transmission and phase-contrast microscopy. The samples were removed from the zero-strain region (cap, FIG. 4C-C), maximum strain region (FIG. 4B-B), and the neck of the construct, where intermediate strains were measured (FIG. 4A-A). Based on the patterns of cellular and fibrillar orientation, it is evident that cellular orientation is influenced by the amplitude of the strain applied onto the construct. The cap region, for example, exhibited very few organized and coherent fibrillar structures when compared with the midsection of the construct. Likewise, randomly oriented cells were observed near the cap of the construct, and almost all the cells were circumferentially oriented in the midsection of the ECCs.

SUMMARY

This study examined the feasibility of creating an engineered cardiac construct made of reconstituted collagen scaffolding and a coculture of smooth muscle cells and neonatal rat cardiomyocytes. The cardiac constructs were cultured dynamically in a multichamber bioreactor and preconditioned with cyclic strain stimulation mim-

icking the wall motion of the native heart muscle. The results show that mechanical preconditioning has a considerable impact on the architectural characteristics of ECCs. Our findings indicate that cellular and morphological reorganization resulting from preconditioning is highly dependent on the amplitude of strain stimulation. Furthermore, this form of preconditioning may be used to compact and align engineered cardiac constructs, thereby increasing the number of cell-to-cell contacts and ultimately improving the structure and function of the tissue substitute.

The study represents a beginning of a necessary detailed characterization of strain amplitude and strain rate effects on cell orientation and collagen fibril architecture in ECCs. We hope to demonstrate the efficacy of mechanical preconditioning as a valuable method of creating myocardial tissue substitutes with superior properties, for physiological coupling with host myocardium.

ACKNOWLEDGMENTS

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