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# Perfusion Improves Tissue Architecture of Engineered Cardiac Muscle

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## ABSTRACT

Cardiac muscle with a certain threshold thickness, uniformity of tissue architecture, and functionality would expand the therapeutic options currently available to patients with congenital or acquired cardiac defects. Cardiac constructs cultured in well-mixed medium had an approximately 100- $\mu$ m-thick peripheral tissue-like region around a relatively cell-free interior, a structure consistent with the presence of concentration gradients within the tissue. We hypothesized that direct perfusion of cultured constructs can reduce diffusional distances for mass transport, improve control of oxygen, pH, nutrients and metabolites in the cell microenvironment, and thereby increase the thickness and spatial uniformity of engineered cardiac muscle. To test this hypothesis, constructs (9.5-mm-diameter, 2-mm-thick discs) based on neonatal rat cardiac myocytes and fibrous polyglycolic acid scaffolds were cultured either directly perfused with medium or in control spinner flasks. Perfusion improved the spatial uniformity of cell distribution and enhanced the expression of cardiac-specific markers, presumably due to the improved control of local microenvironmental conditions within the forming tissue. Medium perfusion could thus be utilized to better mimic the transport conditions within native cardiac muscle and enable *in vitro* engineering of cardiac constructs with clinically useful thicknesses.

## INTRODUCTION

THE REGENERATION OF MYOCARDIUM remains a significant clinical problem, while cardiac transplantation remains limited by the shortage of donor tissue. Novel treatment options, currently under development, include direct injection of stem cells into the scarred myocardium<sup>1-3</sup> and tissue engineering of cardiac muscle.<sup>4-6</sup> Cardiac muscle with a certain minimum thickness, uniformity of tissue architecture, and functionality would expand the therapeutic options currently available to patients with congenital or acquired cardiac defects.

Functional cardiac muscle has been created using isolated neonatal heart cells in conjunction with either

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polymer scaffolds and bioreactors,<sup>4,5,7-9</sup> or collagen gel under tension,<sup>6,10,11</sup> and studied *in vitro* and *in vivo*. However, in all cases, tissue formed only within an approximately 100- $\mu\text{m}$ -thick surface layer, while the construct interior remained largely acellular. Most cells in the surface region displayed myocyte-specific markers.<sup>6,7,9</sup> Constructs were contractile,<sup>6,7</sup> generated tension in response to mechanical, electrical, and chemical stimuli,<sup>5,10,11</sup> and sustained macroscopic signal propagation at rates of 60–400 beats per minute.<sup>5,9</sup> Cardiac constructs based on fetal rat cardiac cells and either alginate gel<sup>12</sup> or gelatine foam<sup>13</sup> survived transplantation and vascularized, but no major improvement of the ventricular function was observed, presumably due to relatively low cellularity of engineered grafts.

The formation of only an outer layer of cardiac-like tissue was consistent with the presence of concentration gradients associated with diffusional mass transport between the construct surfaces and the inner phase. Cardiac myocytes are densely packed and highly metabolically active, therefore nutrients, particularly oxygen, are depleted within a relatively thin layer of viable tissue. Natural myocardium obviates this difficulty through a rich vasculature, with average capillary-to-capillary distances in rat heart of only 17–19  $\mu\text{m}$ , approximately the width of an individual cardiac myocyte.<sup>14-17</sup>

We hypothesized that perfusion can reduce diffusional gradients associated with mass transport over macroscopic distances, improve control of local levels of pH and oxygen, and thereby increase the thickness and spatial uniformity of engineered cardiac muscle. In contrast to, for example, mixed flasks, where mass transport between the culture medium and construct surfaces is enhanced by convection but within the tissue construct occurs by molecular diffusion only, direct perfusion brings culture medium of a desired composition in contact with cells throughout the construct volume. This hypothesis was tested by culturing engineered constructs based on neonatal rat heart cells and biodegradable polymer scaffolds either in cartridges with medium perfusion through the developing tissue, or in control flasks with well mixed medium around the constructs.

## 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.

### *Medium*

DMEM with 4.5 g/L glucose, 10% fetal bovine serum (FBS), 10 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine (all from Gibco, Grand Island, NY), and 100 units/mL penicillin (Sigma, St. Louis, MO).

### *Cell isolation*

Cardiac myocytes were isolated as previously described<sup>7</sup> by digesting ventricles obtained from 1–2-day-old neonatal rats (Taconic, Germantown, NY). Ventricles were quartered, incubated overnight (4°C, 60 rpm) in a 1 mg/mL solution of trypsin (U.S. Biochemicals, Cleveland, OH) in Hank's Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY), washed in culture medium (4 min, 37°C, 60 rpm) and subjected to a series of digestions (3 min, 37°C, 150 rpm) in 1 mg/mL collagenase (type II; Worthington, Freehold, NJ). The first digestate was discarded, and the cell suspensions from the subsequent four to six digestions were centrifuged (1,000 rpm, 4 min), resuspended in 10 mL of HBSS each, pooled, and centrifuged (1,000 rpm, 4 min). The resulting pellet was resuspended in medium and plated in T-75 flasks ( $8 \times 10^6$  cells/mL, 15 mL/flask) for two 75-min periods to enrich the cell suspension with cardiac myocytes by allowing attachment of fibroblasts. Cells that remained unattached were used to seed polymer scaffolds. Approximately 280 rat hearts from 28 different litters were used.

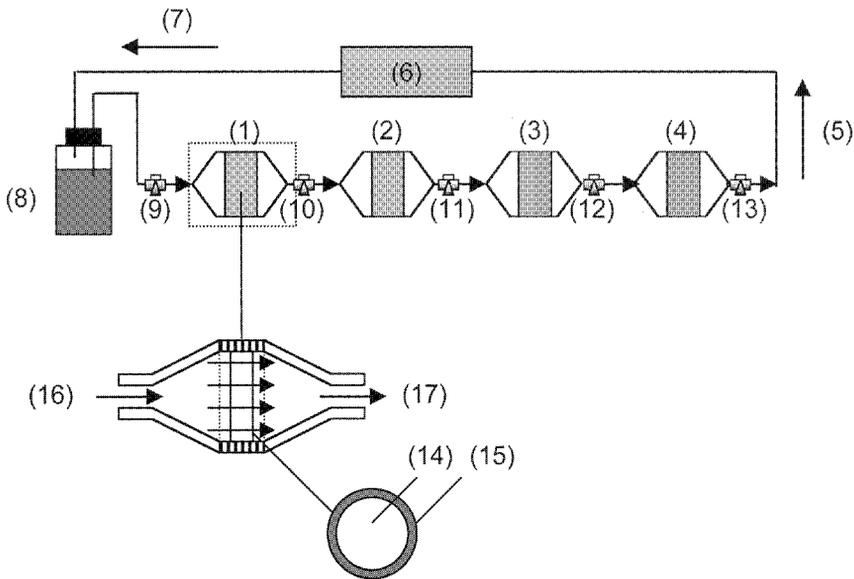
### *Cell seeding of polymer scaffolds*

Discs 1.1-cm in diameter were cut from 2 mm thick nonwoven polyglycolic acid (PGA) mesh (bulk density, 60 mg/cm<sup>3</sup>; fiber diameter, 13  $\mu\text{m}$ ) kindly provided by Smith & Nephew Group Research (York, U.K.). The scaffolds were sandwiched and sealed between two silicone gaskets (1.27/0.95-cm-diameter, 0.5-mm-

thick; Millipore, Bedford, MA) using medical grade silicone adhesive (Dow Corning, Midland, MI), and sterilized by exposure to ethylene oxide. The exposed area of each scaffold had a diameter of 0.95 cm. Scaffolds were prewetted with medium, placed in 35-mm Petri dishes (one per well), and seeded by adding 0.2 mL of cell suspension containing  $24 \times 10^6$  cells. Cell-polymer construct was flipped, any extra cell suspension not absorbed by the scaffold was reapplied, 7 mL of medium were added to each well, and dishes were mounted on an xyz gyator (Shelton, Shelton, CT) set at 24 rpm in a 37°C humidified incubator. After 2 h, unattached cells were reapplied to constructs. After 24 h, medium was replaced, and constructs were flipped. After 48 h, constructs were transferred into perfusion cartridges (one per cartridge) and the perfusion was established as described below.

### Perfusion culture

The perfusion system is depicted in Figure 1. Perfusion cartridges consisted of 13-mm filter holders (Millipore, Bedford, MA). A stainless steel screen (Millipore, Bedford, MA) was placed at the cartridge inlet to disperse flow over the construct surface. A nylon mesh (Nitex, 1-mm pore size; Tetko, Elmsford, NY) was fixed using medical grade silicone adhesive to the silicone gasket on the downstream side of the construct. Cell-polymer constructs were inserted such that the two silicone gaskets sealed together the two halves of the cartridge. The cartridges were arranged in series of four, and each series was connected to a perfusion loop consisting of silicone tubing (0.125/0.25 inch), a medium reservoir, and one channel of a four-channel peristaltic pump (CELLMAX QUAD®; all from Cellco, Germantown, MD). In each loop, 8 ft of silicone tubing (0.125/0.25 inch) was coiled to serve as a gas exchanger. In each pass through the gas exchanger, medium (inside tubing) was equilibrated with incubator air (outside tubing) with respect to partial pressures of oxygen ( $pO_2$ ) and carbon dioxide ( $pCO_2$ ). Perfusion loops were filled with 120 mL of medium



**FIG. 1.** Perfusion system. A series of four cartridges (1–4), each containing one cell-polymer construct, is connected to a silicone-coil gas exchanger (6), a medium reservoir (8), and a peristaltic pump (9). Total volume of culture medium is 120 mL; direction of flow is denoted by arrows (5,7). Medium samples are taken at timed intervals at the inlet and outlet of each cartridge (9–13). A cross section and side view of a perfusion cartridge are shown, with a screen at the inlet to disperse the flow over the construct surface (14) and a screen at the outlet to support the construct (15). The cell-polymer construct (14) is placed between two ring-shaped gaskets (15), to eliminate the flow of medium around the construct and provide perfusion of medium throughout the construct volume (16,17). Only the data for the first construct in each series were included in the present paper.

and operated for 24 h. The series of cartridges was clamped off and drained, each cartridge was opened, a cell-polymer construct transferred in, closed and refilled with medium, and the clamps were removed.

Series of cartridges (one construct per cartridge, four cartridges per series, four series per experiment) were perfused for 1 h at 0.2 mL/min, and then for 10 days at  $3.24 \pm 0.12$  mL/min (nominally 3 mL/min), or  $0.98 \pm 0.02$  mL/min (nominally 1 mL/min), or  $0.62 \pm 0.00$  mL/min (nominally 0.6 mL/min). Medium samples were taken using syringes (Becton Dickinson, Franklin Lakes, NJ) attached to three-way stopcocks (Baxter, Irvine, CA).

### *Spinner flask culture*

Controls for comparing the effects of medium perfusion with the effects of diffusional transport were established for each experiment using spinner flasks (Bellco, Vineland, NJ). Cell-polymer constructs were threaded onto 4-inch-long stainless steel wires (three to four per flask, with 1 disc apiece) embedded into the stopper of a flask and cultured in 120 mL of medium stirred at 50 rpm as previously described.<sup>18</sup> Gas was exchanged via surface aeration of well mixed culture medium around the constructs. Medium samples were taken through the side arms using syringes. Medium was replaced at the same rate as in perfused cartridges (50% or 60 mL every 2 days).

### *Analytical methods*

Medium pH,  $pO_2$ , and  $pCO_2$  were measured daily using a gas analyzer (model 1610; Instrumentation Laboratory, Lexington, MA) with an accuracy of 0.1% for pH and 2% for  $pO_2$  and  $pCO_2$ .<sup>7</sup> The rates of glucose consumption and lactate production were determined from medium concentrations measured using a glucose and L-lactate analyzer (model 2300 STAT Plus; YSI, Yellow Springs, OH).

Construct structure was analyzed using light and transmission electron microscopy. Samples taken for histological analyses ( $n = 2-4$ ) were fixed for 10 min in 4% glutaraldehyde, embedded in paraffin, and cross-sectioned (5- $\mu$ m-thick) through the center of the construct. Sections were stained with hematoxylin and eosin (H&E) for cells and immunohistochemically for cellular expression of sarcomeric  $\alpha$ -actin.<sup>7</sup> Samples for TEM were fixed in Karnovsky's reagent (0.1 M sodium cacodylate with 2% paraformaldehyde and 2.5% glutaraldehyde, pH = 7.4), postfixated in 2% osmium tetroxide, dehydrated in ethanol/propylene oxide, embedded in Poly/Bed812 (Polysciences, Warrington, PA), and sectioned (60-nm-thick). Sections were stained with lead citrate and uranyl acetate and examined with a transmission electron microscope (JEOL-100CX; JEOL, Peabody, MA).

DNA content was measured in homogenates of tissue constructs ( $n = 4$ ) using Hoescht 33258 dye (Polysciences, Warrington, PA) and a spectrofluorometer (Photon Technology International, South Brunswick, NJ) with calf thymus DNA (type 1, highly polymerized; Sigma, St. Louis, MO) as a standard.<sup>19</sup> Protein contents were determined from 100- $\mu$ l samples of homogenates that were assayed as described in the microplate protocol of the BioRad DC Protein Assay kit (BioRad, Hercules, CA).

### *Statistical analysis*

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) in conjunction with Tukey's test for multiple comparisons, using Systat 1.0 for Macintosh.

## RESULTS

### *Cultivation conditions*

For constructs cultured in cartridges, medium was perfused through constructs at flow rates of 0.6–3.0 mL/min (corresponding to 0.8–4.2  $cm^3/cm^2 \cdot min$ , or 140–700  $\mu m/sec$ ) and equilibrated in each pass through gas exchanger, such that the inlet concentrations of oxygen and carbon dioxide were maintained at constant levels (Fig. 1). For constructs cultured in spinner flasks, medium was in contact with the outer construct surfaces and equilibrated with incubator air with surface aeration. As compared to flask cultures, medium

PERFUSION OF ENGINEERED CARDIAC MUSCLE

TABLE 1. MEASURED VALUES OF METABOLIC PARAMETERS IN CULTURE MEDIUM FROM PERFUSED CARTRIDGES AND MIXED FLASKS

	Group	Parameter level at the construct inlet	Parameter change across the construct
pH	Flask	7.24 ± 0.02 <sup>a</sup>	n/a
	0.6 mL/min	7.34 ± 0.02	0.010
	1 mL/min	7.34 ± 0.01	0.006
	3 mL/min	7.34 ± 0.01	0.007
pO <sub>2</sub> (mm Hg)	Flask	130 ± 4 <sup>a</sup>	n/a
	0.6 mL/min	157 ± 2	10
	1 mL/min	156 ± 1	10
	3 mL/min	157 ± 2	6
pCO <sub>2</sub> (mm Hg)	Flask	49.7 ± 0.3 <sup>a</sup>	n/a
	0.6 mL/min	39.6 ± 0.5	1.4
	1 mL/min	39.2 ± 0.4	1.2
	3 mL/min	39.3 ± 0.9	1.2
Glucose (mg/dL)	Flask	332 ± 10	n/a
	0.6 mL/min	346 ± 11	1
	1 mL/min	352 ± 13	0
	3 mL/min	348 ± 18	0
Lactate (mg/dL)	Flask	80.0 ± 10	n/a
	0.6 mL/min	79.0 ± 10	0.6
	1 mL/min	71.9 ± 11	0.3
	3 mL/min	72.5 ± 18	0.1

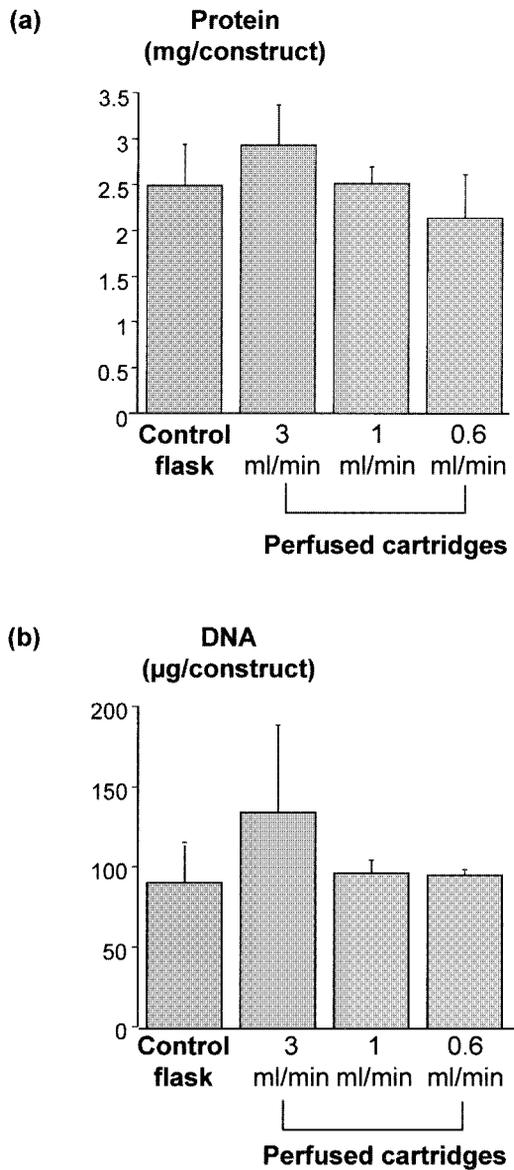
<sup>a</sup>Statistically significant difference when compared to all other groups ( $p < 0.05$ ).

in perfused cartridges had a higher pO<sub>2</sub>, higher pH, and lower pCO<sub>2</sub> ( $n = 4-7$ ;  $p < 0.001$ ); glucose and lactate levels were comparable (Table 1). The changes in medium composition across each cartridge, estimated by linear extrapolation of measured values at the inlet and outlet of the series, were relatively small (Table 1), such that the microenvironmental conditions could be considered to be relatively uniform throughout the construct volume. In contrast, concentration gradients within constructs were inherent to flask cultures. An analysis of diffusional oxygen transport within flask constructs showed that the oxygen tension would be expected to decrease to zero at an estimated depth from the construct surface of 99 μm (i.e., only 5% of the total thickness; Appendix 1).

The hydrodynamic shear stresses acting on the cells within perfused constructs were estimated to be 1.0 ± 0.1, 0.30 ± 0.002, and 0.19 ± 0.02 dyn/cm<sup>2</sup> for perfusion rates of 3, 1, and 0.6 mL/min, respectively (Appendix 2).

### Cell number and distribution

The DNA and protein contents of constructs cultured for 10 days in perfused cartridges and spinner flasks were comparable, indicating comparable total cell number (Fig. 2). Protein/DNA ratios were 38.5 ± 9 mg/mg ( $n = 9$ ) and 39.5 ± 5 mg/mg ( $n = 7$ ) for perfused and flask constructs, respectively. However, perfusion markedly improved the uniformity of cell distribution. In flask constructs, cells were concentrated mostly within an approximately 100-μm-thick outer region, while the inner construct volume was largely acellular. In perfused constructs, the spatial cell distribution was markedly improved (Fig. 3a). Image processing of construct histological cross sections demonstrated that cell number per unit area was relatively constant

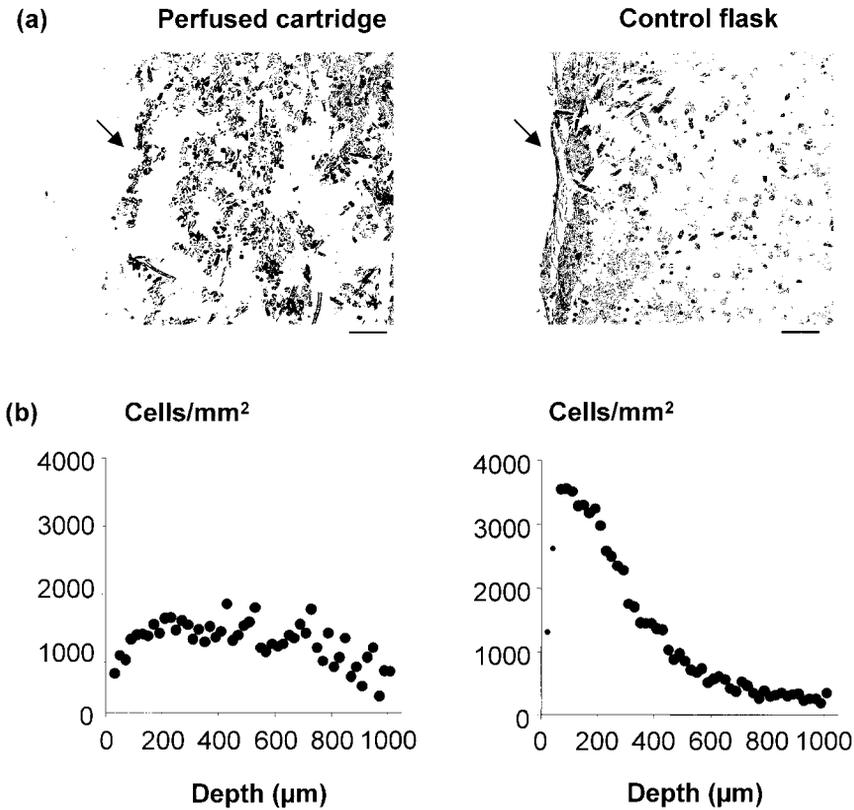


**FIG. 2.** (a) Protein content and (b) DNA content of the constructs cultured for 10 days in cartridges (perfused at 3, 1, or 0.6 mL/min) or control flasks (stirred at 50 rpm).

within perfused constructs, but decreased sharply as a function of the depth for flask constructs (Fig. 3b). The cell density measured over the entire cross-sectional area was  $1,160 \pm 316$  cells/mm<sup>2</sup> ( $n = 12$ ) and  $1,335 \pm 1,119$  cells/mm<sup>2</sup> ( $n = 36$ ) for perfused and flask constructs, respectively. Therefore, although the average values were comparable for the two groups, the rates of change in local values were distinctly different, as evidenced by the 3.5-fold higher standard deviation for flask then perfused constructs.

#### *Cell phenotype*

Perfused constructs contained a relatively uniform population of cells expressing sarcomeric  $\alpha$ -actin (Fig. 4a,b), a protein whose expression is indicative of a differentiated muscle cell phenotype, whereas in flask constructs this protein was expressed mostly by cells located within a 100- $\mu$ m-thick region at the construct surface, and only occasionally by cells in the construct interior (Fig. 4c,d). In perfused constructs, multiple



**FIG. 3.** Cell distributions within perfused and flask constructs. Data are shown for constructs cultured for 10 days in perfused cartridges at 0.6 mL/min or stirred flasks at 50 rpm. (a) Histomorphology (arrow denotes the construct edge; bar = 100 μm) and (b) Two-dimensional cell density versus distance from the construct surface.

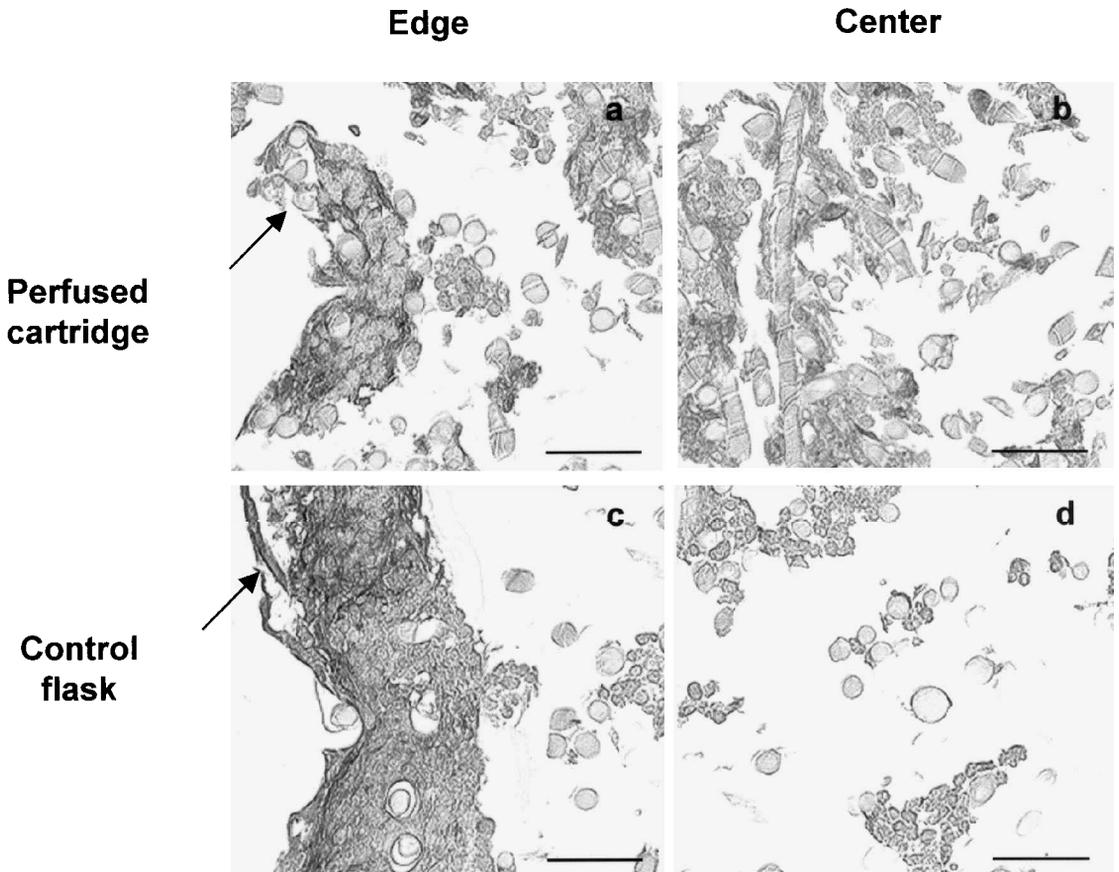
layers of elongated cells were observed among groups of rounded cells throughout the construct volume (Fig. 4a,b), whereas in flask constructs, most of the elongated cells were at the construct edges (Fig. 4c,d).

Transmission electron microscopy demonstrated improved structure of perfused as compared to flask constructs (Fig. 5). Perfused constructs contained cardiac myocytes expressing differentiated ultrastructure (distinct and well-formed sarcomeres, junctions characteristic of myocardial intercalated discs), both at the edges and centers (Fig. 5a,b). In contrast, flask constructs contained cells with organized sarcomeres and well formed junctions at edges (Fig. 5c), but cells near the center had less well organized sarcomeres, less numerous and less well formed cell junctional complexes, and less dense cytoplasm with fewer recognizable organelles (Fig. 5d). Cells throughout perfused constructs and at the edges of flask constructs contained abundant glycogen granules, suggesting aerobic metabolism; the centers of flask constructs contained very few cells, and most of these showed signs of necrosis.

## DISCUSSION

A major difficulty in engineering most tissue types is that native tissues are vascularized, whereas engineered tissues are not.<sup>20</sup> As a result, engineered tissues that are more than approximately 100-μm-thick and highly metabolically active (e.g., heart muscle, liver, pancreatic islets) and supplied with nutrients solely by diffusion may have insufficient transport to and from the cells.<sup>21–24</sup> Enhanced mass transport at tissue surfaces, for example by construct settling in dynamic laminar flow, can improve the properties of engineered tissues by improving mass transport at construct surfaces.<sup>7,9,25,26</sup>

Perfusion systems have been considered for a variety of engineered tissues, including skeletal muscle<sup>27</sup>



**FIG. 4.** Cellular expression of sarcomeric  $\alpha$ -actin assessed immunohistochemically at the edge (denoted by arrows; **a,c**) and center (**b,d**) of constructs cultured for 10 days in perfused cartridges (0.6 mL/min) or control flasks (50 rpm). Bar = 50  $\mu$ m.

and cartilage,<sup>28–31</sup> but without eliminating the flow of medium around the constructs and providing true perfusion of medium throughout the construct volume. The present study was designed to explore direct perfusion of engineered cardiac muscle with culture medium as a new paradigm for cardiac tissue engineering, and a means to reduce concentration gradients of nutrients and metabolites within the developing tissue. Improved control of microenvironmental conditions within an engineered tissue could potentially enable *in vitro* creation of thick tissue-like patches for use in pharmacological and physiological studies *in vitro* and tissue repair studies *in vivo*.

Medium metabolic parameter levels in perfused and flask cultures differed in two ways. As compared to flask constructs, cells in perfused constructs were exposed to (1) higher  $pO_2$  and pH and lower  $pCO_2$ , and (2) more homogenous microenvironmental conditions. For flask constructs, cultured under conditions of diffusional mass transport, the estimated thickness of tissue over which oxygen was completely depleted from the medium was approximately 100  $\mu$ m. This value agreed well with histological data showing an ap-

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**FIG. 5.** Cellular ultrastructure as viewed by transmission electron microscopy of constructs cultured for 10 days in (**a,b**) perfused cartridges (0.6 mL/min) and (**c,d**) spinner flasks (50 rpm), at the surface depths of 0.1 mm (**a,c**) and 0.5–1 mm (**b,d**). Arrow in **a** and **c** shows a well-formed junctional complex; **a**, **b**, and **c** show a healthy, differentiated phenotype, but **d** shows poorly formed sarcomeres, lack of well-formed cell junctions, and little if any glycogen. All originally 10,000 $\times$ . Bar = 1  $\mu$ m. Representative images of tissue samples are shown for both groups.

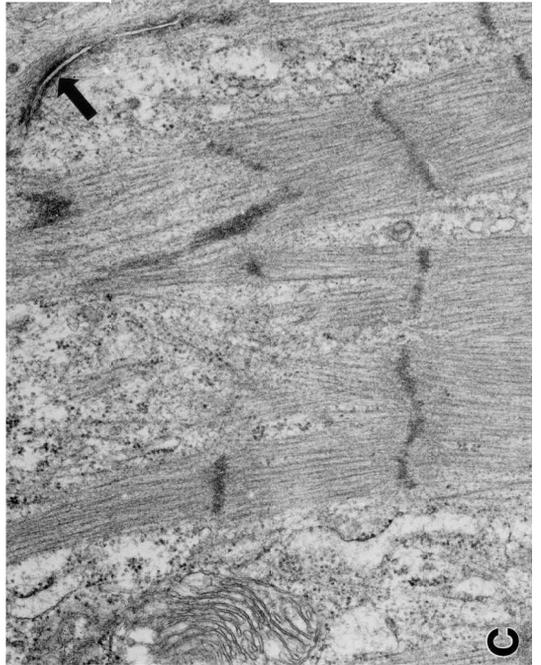
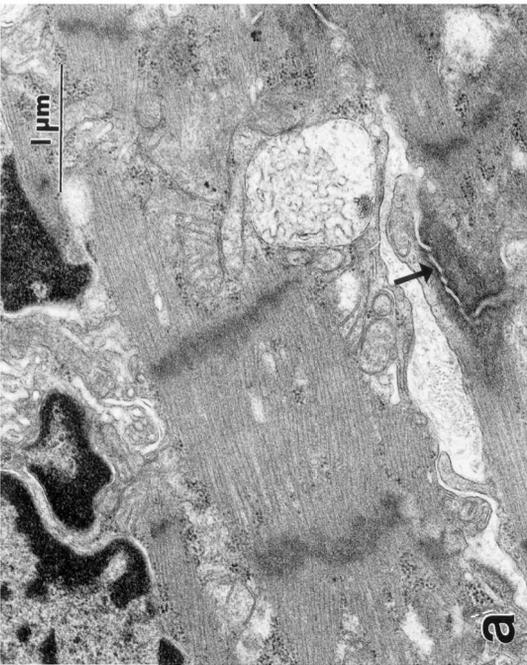
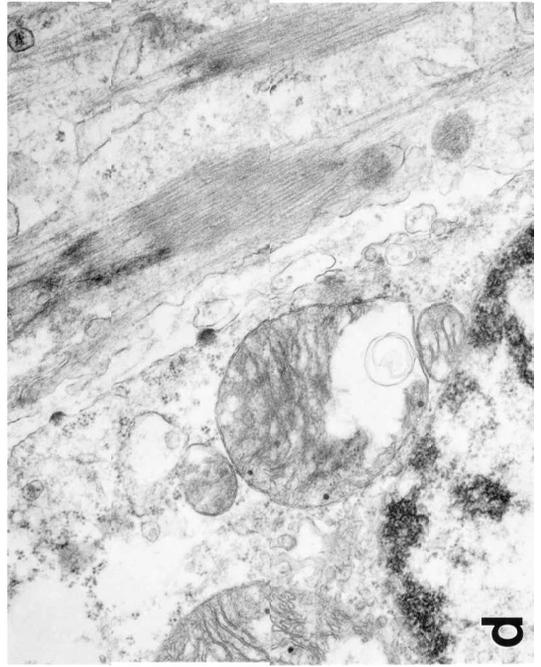
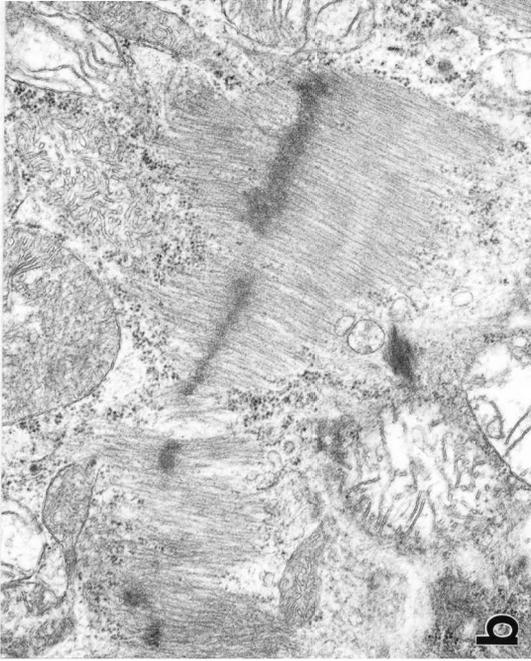


FIG. 5.

proximately 100- $\mu\text{m}$ -thick capsule of continuous tissue at the construct surface and a relatively cell-free interior (Fig. 3a).<sup>5,7,9</sup> Moreover, 100  $\mu\text{m}$  is also the approximate thickness of the zone of subendocardium that survives a myocardial infarct, owing to perfusion from blood in the left ventricular cavity.<sup>32</sup>

The control over the cell microenvironment was much better in perfused than in flask cultures, due to the direct flow of medium throughout the construct rather than only at construct surfaces. Exposure to more homogenous environmental conditions within perfused constructs was associated with significant improvement in the uniformity of the cell distribution across the entire construct thickness (Fig. 3). In spite of differences in cell distribution, the total cell numbers were comparable in perfused and flask constructs (Fig. 2), presumably due to the same initial number of seeded cells. In flasks, the cell concentration was high at construct surfaces, that is, in the region of high oxygen concentration, in contrast to perfused constructs where both the cells and oxygen were relatively uniformly distributed throughout the construct volume. Perfusion also enhanced the expression of a differentiated cell phenotype within cardiac constructs, and resulted in a relatively uniform population of cardiac myocytes showing cardiac-specific markers (e.g., expression of sarcomeric  $\alpha$ -actin, sarcomeres, well formed cell junctions, elongated morphology) and evidence of aerobic metabolism (glycogen granules). In flask constructs, expression of these markers of differentiation and overall cell well-being were limited to a 100  $\mu\text{m}$  thick region at construct surfaces (Fig. 4c,d).<sup>5,7,9</sup>

The fact that most cells within perfused cardiac constructs exhibited rounded phenotype may have been due to shear. One response of various cell types to shear is rounding.<sup>33</sup> In spite of the fact that the estimated shear forces within perfused constructs were relatively low (0.19–1.04 dyn/cm<sup>2</sup>; Appendix 2), slightly higher shear stresses (i.e., 1.6–3.3 dyn/cm<sup>2</sup>) applied *in vitro* over extended periods of time altered the morphology and decreased the viability of various mammalian cell types, including hybridoma and human embryonic kidney cells.<sup>34,35</sup> The response to shear stress depends upon many factors, including cell type, exposure time, substrate, and flow dynamics.<sup>33,35</sup> Cardiac cells are not exposed to direct shear forces *in vivo*, and the exchange of nutrients and metabolic wastes is carried out by diffusion between densely spaced capillaries and the cells.

The present study demonstrated that perfusion can markedly and significantly improve the uniformity of the engineered tissue. Recent *in vivo* studies of engineered cardiac grafts<sup>12,13</sup> showed promising results with respect to construct survival, vascularization and integration, but the functional improvement was either not observed<sup>13</sup> or was not significant.<sup>12</sup> Future work is thus expected to be driven by the need to create thicker grafts with more uniform tissue architectures for controlled *in vitro* and *in vivo* studies. In particular, there is a need to increase of the initial cell concentration, and to coculture the endothelial with cardiac muscle cells in order to compartmentalize nutrient supply and waste removal in a more physiological way, and to enhance cell survival during *in vitro* cultivation and *in vivo* implantation.

To summarize, cultivation with direct perfusion of medium exposed engineered cardiac tissue to more homogeneous conditions and maintained higher oxygen tension in the cell microenvironment, as compared to spinner flasks. Perfused constructs had relatively uniform spatial distributions of cells expressing differentiated phenotype. Medium perfusion can thus be utilized to enhance mass transport to and from the cells and allow the cultivation of engineered tissues >100  $\mu\text{m}$  in thickness.

## ACKNOWLEDGMENTS

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## APPENDIX 1: CALCULATION OF OXYGEN GRADIENTS FOR CONSTRUCTS CULTURED IN FLASKS

A one-dimensional analysis of the diffusional oxygen transport through a flask-cultured construct was used to estimate the depth,  $l$ , from the construct surface at which oxygen tension decreased to zero. The

construct was modeled as a flat slab using the following simplifying assumptions: (1) constant rate of cellular oxygen consumption,  $R_v$ , (2) constant diffusivity of oxygen,  $D$ , (3) steady state conditions, (4) negligible convective flow through the construct, and (5) constant oxygen concentration,  $C_o$ , at the construct surface, equal to the bulk medium oxygen concentration due to convective flow and mixing. Under these conditions, the conservation equation for oxygen involves diffusion and consumption terms:

$$D * d^2C/dx^2 + R_v = 0 \quad (1)$$

where  $x$  is the depth from the construct surface and  $x = 0$  refers to the construct surface. Equation 1 was solved using the following boundary conditions:

$$C_{x=0} = C_o \text{ (assumption 5)} \quad (2)$$

$$dC/dx_{x=L} = 0 \quad (3)$$

to obtain the oxygen concentration profile:

$$C = -R_v/D * (-x^2/2 + L * x) + C_o \quad (4)$$

$L$  was estimated from Equation 4 using the following parameter values:

$$C_o = 130 \text{ mm Hg } (2.04 \times 10^{-7} \text{ moles/mL})$$

$$D = 2.0 \times 10^{-5} \text{ cm}^2/\text{sec}$$

$R_v = 5.04 \times 10^{-6} \text{ mol O}_2/\text{min}/\text{cm}^3$ , based on an oxygen consumption rate of  $3.0 \times 10^{-8} \text{ mol/mg protein/min}$ ,<sup>37</sup> a cellular protein content of  $8.4 \times 10^{-7} \text{ mg/cell}$ ,<sup>38</sup> and a cell density of  $2.0 \times 10^8 \text{ cells/cm}^3$ .<sup>36</sup>

## APPENDIX 2: CALCULATION OF THE SHEAR STRESS WITHIN PERFUSED CONSTRUCTS

Medium was perfused through cardiac constructs at flow rates of 0.6–3.0 mL/min (corresponding to 0.8–4.2 cm<sup>3</sup>/cm<sup>2</sup> min per construct cross-sectional area, or 140–700 μm/sec). Shear stress acting on cells within a perfused construct was estimated by equating the average energy dissipation with the drag force per unit surface area of a polymer fiber coated with cells:<sup>39</sup>

$$\tau_{av} = F_d/S \quad (5)$$

where  $\tau_{av}$  = the average shear stress on the cell surface,  $F_d$  = drag force, and  $S$  = surface area of polymer fiber. Drag force is equal to the pressure drop across a cell-polymer construct multiplied by its cross-sectional area,  $A$ , which can be calculated as:

$$A = V/L \quad (6)$$

where  $L$  = the total length of perfused tissue in the direction of flow and  $V$  = the volume of the perfused construct. By substitution, Equation 5 becomes:

$$\tau_{av} = -\Delta P/L * 1/(S/V) \quad (7)$$

where  $\Delta P$  = the pressure drop across the perfused tissue. The Reynold's number ( $Re$ ) within the construct was estimated using:

$$Re = U_o * d_f * \rho/\mu \quad (8)$$

$$U_o = Q/A \quad (9)$$

where  $U_o$  is the superficial fluid velocity within the construct;  $d_f$  = diameter of polymer fibers coated with cells ( $d_f = 23 \mu\text{m}$  assuming a  $5\text{-}\mu\text{m}$  thick cell layer<sup>39</sup> on  $13\text{-}\mu\text{m}$ -diameter fibers); medium density of  $\rho = 1.03 \text{ g/cm}^3$ ;<sup>40</sup> medium viscosity of  $\mu = 0.7 \text{ cP}$  ( $0.0007 \text{ Ns/m}^2$ ) at  $37^\circ\text{C}$ <sup>39</sup>; the volumetric flow rate of medium of  $Q = 0.62\text{--}3.24 \text{ mL/min}$ ; and the construct cross sectional area  $A = 0.712 \text{ cm}^2$ . Equation 8 was solved to yield  $Re = 0.005\text{--}0.026$ . Because  $Re < 10$  at all conditions, the construct could be considered as an isotropic porous medium, and Darcy's Law could be applied<sup>41</sup>:

$$\Delta P/L = \mu U_o/k \quad (10)$$

where  $k$  = permeability, a property which depends on the size, concentration, and arrangement of the fibers in a fibrous medium. After substituting Equation 10 into Equation 7 and expressing  $S/V$  as a function of the construct void fraction,  $\epsilon$ , and fiber diameter,  $d_f$ , Equation 7 becomes:

$$\tau_{ave} = \mu * U_o/k * d_f/\{4 * (1 - \epsilon)\} \quad (11)$$

$\epsilon$  was calculated from the volume fractions of cells and polymer fibers. The volume fraction of cells was estimated using the measured DNA content of each construct, a measured value of  $3.05 \times 10^{-5} \mu\text{g DNA/cell}$ , and a cell volume of  $1.77 \times 10^{-9} \text{ cm}^3$ .<sup>42</sup> The volume fraction of polymer fibers was previously determined to be 3%.<sup>43</sup> The resulting values of  $\mu$  for constructs perfused at 3, 1, and 0.6 mL/min were 0.921, 0.929, and 0.934, respectively. The corresponding  $k$  values were estimated based the permeability of a random fibrous medium expressed as a function of fiber diameter and the volume fraction of the fibers, as previously suggested,<sup>41,44</sup> to obtain 3.8, 4.2, and 4.8 for constructs perfused at 3, 1, and 0.6 mL/min, respectively. The average hydrodynamic shear  $\tau_{av}$  was estimated from Equation 11 to be  $1.04 \pm 0.11$ ,  $0.30 \pm 0.002$ , and  $0.19 \pm 0.02 \text{ dyn/cm}^2$  for the flowrate of 3, 1, and 0.6 mL/min, respectively.

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