Medium perfusion enables engineering of compact and contractile cardiac tissue

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Radisic, Milica, Liming Yang, Jan Boublik, Richard J. Cohen, Robert Langer, Lisa E. Freed, and Gordana Vunjak-Novakovic. Medium perfusion enables engineering of compact and contractile cardiac tissue. Am J Physiol Heart Circ Physiol 286: H507–H516, 2004.—We hypothesized that functional constructs with physiological cell densities can be engineered in vitro by mimicking convective-diffusive oxygen transport normally present in vivo. To test this hypothesis, we designed an in vitro culture system that maintains efficient oxygen supply to the cells at all times during cell seeding and construct cultivation and characterized in detail construct metabolism, structure, and function. Neonatal rat cardiomyocytes suspended in Matrigel were cultured on collagen sponges at a high initial density (1.35 × 10⁶ cells/cm³) for 7 days with interstitial flow of medium; constructs cultured in orbitally mixed dishes, neonatal rat ventricles, and freshly isolated cardiomyocytes served as controls. Constructs were assessed at timed intervals with respect to cell number, distribution, viability, metabolic activity, cell cycle, presence of contractile proteins (sarcomeric α-actin, troponin I, and tropomyosin), and contractile function in response to electrical stimulation [excitation threshold (ET), maximum capture rate (MCR), response to a gap junctional blocker]. Interstitial flow of culture medium through the central 5-mm-diameter × 1.5-mm-thick region resulted in a physiological density of viable and differentiated, aerobic metabolizing cells, whereas dish culture resulted in constructs with only a 100- to 200-μm-thick surface layer containing viable and differentiated but anaerobically metabolizing cells around an acellular interior. Perfusion resulted in significantly higher numbers of live cells, higher cell viability, and significantly more cells in the S phase compared with dish-grown constructs. In response to electrical stimulation, perfused constructs contracted synchronously, had lower ETs, and recovered their baseline function levels of ET and MCR after treatment with a gap junctional blocker; dish-grown constructs exhibited arrhythmic contractile patterns and failed to recover their baseline MCR levels. 

Tissue culture. Cardiomyocytes were obtained from 1- to 2-day-old neonatal Sprague-Dawley (Charles River) rats according to procedures approved by the Institutional Committee on Animal Care, as previously described (5). Porous collagen scaffolds (Ultrafoam, have been engineered in vitro starting from cardiac myocytes in conjunction with collagen gels (9, 11, 34, 35), collagen fibers (2), collagen sponges (15, 19, 20, 25, 28), and polyglycolic acid meshes (3, 5, 24) or by stacking confluent cell monolayers into thin pulsatile sheets (26). In some cases, in vitro cultivation involved the use of bioreactors (3, 5, 24) or the application of mechanical stretch (1, 9, 36). In all cases, oxygen transport through the tissue was largely governed by molecular diffusion, which can support only a thin (100–200 μm) surface layer of functional tissue, and leaves construct interior relatively acellular (5, 35).

Construct cultivation in perfused cartridges markedly improved the uniformity of cell distribution, but the overall cell density remained low due to the limitations of diffusional oxygen supply during scaffold seeding (6, 7). In a recent study, we showed that gel inoculation, in conjunction with interstitial flow of culture medium, enables rapid seeding of hypoxia-sensitive cells (C2C12 cell line) at high and spatially uniform initial densities and evaluated the feasibility of the proposed method for seeding and cultivation of cardiomyocytes (25). Seeding in perfusion yielded a spatially uniform physiological cell density, whereas dish-seeded constructs contained viable cells only within a thin (100–200 μm) surface layer (25).

On the basis of these previous studies, we hypothesized that thick, compact, and functional cardiac constructs could be engineered in vitro by providing convective-diffusive oxygen transport similar to that present in vivo between the capillary network and the cells within native myocardium. To test this hypothesis, we established a culture system that maintained efficient oxygen supply to the cells at all times during scaffold seeding and construct cultivation and characterized in detail construct metabolism, structure, and function. Neonatal rat cardiomyocytes were suspended in Matrigel, inoculated into collagen sponges at a physiological cell density, and cultured for 7 days. Medium perfusion through cell-seeded scaffolds was established within 10 min of cell inoculation and maintained throughout the duration of construct cultivation at an interstitial velocity of 500 μm/s, corresponding to the average blood velocity within native myocardium (12).

METHODS

Tissue culture. Cardiomyocytes were obtained from 1- to 2-day-old neonatal Sprague-Dawley (Charles River) rats according to procedures approved by the Institutional Committee on Animal Care, as previously described (5). Porous collagen scaffolds (Ultrafoam,
Davol) formed as 11-mm-diameter × 1.5-mm-thick disks were inoculated at the density of 1.35 × 10⁶ cells/cm³ scaffold volume. Cells were suspended in Matrigel (Becton-Dickinson) using 12 10⁶ cells in 60 μl Matrigel, delivered into scaffolds, and transferred immediately after gelation into either orbitally mixed dishes or perfused cartridges as previously described (25).

In the perfused group, constructs were placed between two stainless steel screens and two silicone gaskets and transferred into 1.5-ml polycarbonate perfusion cartridges (kindly donated by Advanced Tissue Sciences; La Jolla, CA; 1 scaffold/cartridge). The screens (85% open area) provided mechanical support during perfusion, and the gaskets (1 mm thick, 10 mm outer diameter, 5 mm inner diameter) routed the culture medium through the central area of the construct. Constructs were subjected to alternating flow perfusion at 0.5 ml/min for the first 1.5 h to prevent washout of cardiomyocytes before they attached to the scaffold (Fig. 1A) and then to unidirectional perfusion for the duration of culture (an additional 7 days) (Fig. 1B). Control constructs were cultivated in orbitally mixed dishes (25 rpm).

Culture medium was DMEM (4.5 g/l glucose) supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, and 100 U/ml penicillin (all from GIBCO-BRL).

**Flow visualization.** Fluid flow in the perfused cartridge fitted with the Ultrafoam scaffold was evaluated by introducing a pulse of methylene blue tracer dye (Sigma) into the inlet flow of distilled water. The flow velocity was set 500 μm/s. In separate experiments, a thin stream of red flow tracer dye (VWR; Bridgeport, NJ) was injected isokinetically 3 mm upflow of the scaffold to evaluate any dissipation of fluid flow across the cartridge. High resolution images were taken using a digital camera (Nikon Coolpix 900).

**Analytic methods.** Constructs and culture medium were sampled immediately postseeding and after 1.5 h, 1 day, and 7 days of culture. Cell number, viability, metabolism [lactate yield on glucose (L/G)],

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**A: Scaffold seeding**

![Diagram of scaffold seeding](image)

**B: Construct cultivation**

![Diagram of construct cultivation](image)

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Fig. 1. Perfused cartridges with interstitial flow of culture medium. A: scaffold seeding. Gel-cell inoculated scaffolds (9) were placed between two stainless steel screens (7) and two silicone gaskets (8) and transferred into cartridges (3; one scaffold per cartridge). The screens (85% open area) provided mechanical support during perfusion, and the gaskets (1 mm thick, 10 mm outer diameter, 5 mm inner diameter) routed the culture medium directly through the construct. Air bubbles were displaced by culture medium injected from the debubbling syringe (4) into the downstream syringe (2). Each cartridge was placed in a perfusion loop consisting of a push-pull pump (6), two gas exchangers (1 and 5), and two syringes (2 and 4). The total volume of medium was 8 ml. For a period of 1.5 h, the pump was programmed to a flow rate of 0.5 ml/min, with the reversal of flow direction after 2.5 ml was perfused in a given direction. Inset, flow configuration in perfused constructs. B: construct cultivation. Each cartridge with a seeded construct (4) was transferred into a perfusion loop consisting of one channel of a multichannel peristaltic pump (1), gas exchanger (a coil of thin silicone tubing, 3 m long) (2), reservoir bag (3), and two syringes (5 and 6). The total volume of medium in each loop was 32 ml. The flow rate was set at 0.5 ml/min (corresponding to the interstitial velocity of 500 μm/s) and maintained throughout the 7-day cultivation. Constructs seeded and cultivated in orbitally mixed dishes (35-mm well, 8 ml medium, 25 rpm) served as controls. C: flow visualization. Top, bulk flow through the construct center; bottom, stream of tracer dye. Scale bar = 10 mm.
and cell cycle were assessed at all time points; cell distribution, the presence of contractile proteins, and contractile function were assessed at 7 days. A total of 26 rat litters (~260 heart ventricles) was used in 10 independent experiments, with n = 2–8 constructs/data point.

Cell number and viability. Cell number and viability were assessed using ethidium monoazide bromide (EMA) in conjunction with fluorescence-activated cell sorting (FACS) as previously described (25). In brief, EMA was added to aliquots of freshly isolated neonatal rat cardiomyocytes or constructs (10 µl of 50 µg/ml solution per 1 × 10⁶ cells suspended in 100 µl PBS) in six-well dishes that were placed on ice under fluorescent light for 10 min to allow for EMA to cross-link to DNA of nonviable cells. EMA-labeled constructs were digested with collagenase and dispase (10 ml of solution containing 0.6 mg/ml collagenase type II with 282 U/mg and 1.2 U/ml of dispase in culture medium per construct) for 30 min at 37°C and 30 min on ice, with periodic pipetting to dissociate cell aggregates. EMA-labeled dissociated cells were rinsed with culture medium and counted using a hemocytometer (VWR). Cells were resuspended in PBS at the concentration of 10⁶ cells/ml and subjected to FACS (FACScan, Becton-Dickinson). The change in cell viability was calculated as the difference between the measured viabilities of freshly isolated cells and cells harvested from digested constructs. As an independent measure of cell damage and death, a lactate dehydrogenase (LDH) assay was performed on samples of culture medium after 1, 3, and 7 days of culture using a commercial kit (Chiron Diagnostics; East Walpole, MA), as described previously (5).

Cell metabolism. Cell metabolism was assessed from the molar ratio of lactate produced to glucose consumed (L/G; ideally, 1 mol/mol for aerobic metabolism and 2 mol/mol for anaerobic metabolism). Glucose and lactate concentrations were measured in culture medium sampled at timed intervals using a glucose and l-lactate analyzer (model 2300 STAT Plus, Yellow Springs Instruments; Yellow Springs, OH).

Cell cycle analysis. For cell cycle analysis, constructs were digested, and cells were permeabilized in 70% ethanol (1 ml/10⁶ cells) for 30 min at 4°C. After centrifugation (10 min, 1,000 rpm), the pellet was resuspended in a solution of 50 µg/ml RNase A and 0.1% Triton X-100 in PBS (0.5 ml/10⁶ cells) to digest double-stranded RNA, which might interfere with staining. Propidium iodide was added (50 µg/ml solution per 10⁶ cells) to digest double-stranded RNA, and cell suspension was subjected to FACS (FACScan, Becton-Dickinson) to determine the fraction of cells in the G0/G1, S, and G2/M phases. Peak deconvolution was performed using ModFit LT V2.0 for Macintosh.

Histological evaluation. For histological evaluation, constructs were paraffin embedded, bisected, cross sectioned (5 µm thick), and either stained with hematoxylin and eosin or immunostained as previously described (5) with monoclonal antibodies for sarcomeric α-actin (diluted 1:500, Sigma), cardiac troponin I (diluted 1:150, Biodesign), or sarcomeric tropomyosin (diluted 1:100, Sigma).

Contractile function. The contractile function of engineered cardiac constructs was evaluated by monitoring contractile activity upon electrical stimulation at ×10 magnification using a microscope (Nikon Diaphot). Each construct was placed in a 100-mm petri dish containing 120 ml Tyrode solution (140 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, and 5.5 mM d-glucose; pH 7.4) between two custom-made gold electrodes connected to a cardiac stimulator (Nihon Kohden). The temperature was maintained at 37°C using a heating tape (VWR) attached to the bottom of the petri dish. The stimuli (square pulses, 2-ms duration) were applied at a rate of 60 beats/min starting at an amplitude of 1 V, which was gradually increased in 0.1-V increments until the excitation threshold (ET) was reached and the entire construct was observed to beat synchronously.

The maximum capture rate (MCR) was defined as the maximum pacing frequency (in beats/min) for a synchronous stimulus response of the constructs. To measure MCR, the stimulation frequency was increased (from 60 to 600 beats/min, in 20 beats/min increments) at a constant voltage (equal to either 150% or 200% of the ET). The construct response was measured at each frequency, and the frequency was increased until the contractions became asynchronous, irregular, or completely ceased. The maximum frequency for synchronous contractions was recorded as the MCR. The value of ET, which was different from one construct to another due to the differences in their structures, served as the baseline voltage for the measurement of MCR. One example of the recording sequence is shown in Radsscic video 3 (see http://ajpheart.physiology.org/cgi/content/full/00171.2003/DC1).

RESULTS

Cell number and viability. Cell survival was consistent with the conditions of flow in each experimental group. Flow visualization demonstrated that the central 5-mm-diameter region of the perfused constructs had uniform interstitial flow, whereas the outer edge ring between the two gaskets was shielded from flow (Fig. 1). In orbitally mixed dishes, fluid flow was generated at construct surfaces but not in their interiors.

The effects of culture system and time of culture on cell survival and function are shown in Fig. 2; the corresponding statistical data are shown in Table 1. In seeded constructs (1.5-h time point), the live cell numbers were comparable for perfused and control constructs (~7 million cells/construct in both groups; Fig. 2A). Throughout the cultivation (1- and 7-day time points), the number of live cells in perfused constructs was significantly higher than that in dish-grown constructs (Fig. 2A). Notably, the number of live cells in dish-grown constructs decreased rapidly during the first day of culture and continued to decrease relatively slowly between days 1 and 7. In contrast, live cell numbers in perfused constructs did not change from 1.5 h to day 1 and decreased slowly over the time of culture. The volume fraction of the construct that was adequately perfused corresponded roughly to the fraction of cells that remained viable over 7 days of culture (Fig. 2A). Cell viability was significantly higher in perfused than dish-grown constructs at all time points (Fig. 2B). Importantly, the final cell viability in perfused constructs (81.6 ± 3.7%; Fig. 2B) was not significantly different from the viability of the freshly isolated cells (83.8 ± 2.0%), and it was markedly and signif-
significantly higher than the cell viability in dish-grown constructs (47.4 ± 7.8%; Fig. 2B). The overall level of cell damage and death was assessed by monitoring the levels of LDH activity in the culture medium. At all time points tested (1, 3, and 7 days), the levels of LDH were significantly lower in perfused constructs compared with the orbitally mixed dish (Fig. 2E), indicating less cell damage and death.

**Cell metabolism.** The Po2 in culture medium was significantly higher at the inlet of perfusion cartridges than in orbitally mixed dishes (145 ± 1 vs. 135 ± 1 mmHg, P < 0.05, n = 5–8). The measured decrease in oxygen tension across the perfused cartridge was only 8 mmHg, suggesting that culture medium rich in oxygen was available to the cells throughout the construct volume. In contrast, culture medium rich in oxygen was available only to the cells at surfaces of dish-grown constructs. The molar ratio of lactate produced to glucose consumed (L/G) was ~1 for perfused constructs throughout the duration of culture, indicating aerobic cell metabolism.
metabolism (Fig. 2C). In contrast, L/G increased progressively from 1 to ~2 with time of culture for dish-grown constructs, indicating a transient to anaerobic cell metabolism (Fig. 2C). The increase in L/G with time in dish cultures was statistically significant ($P < 0.001$). The consumption rate of glucose, the primary energy source in our system, per unit live cells was not significantly different between the groups at any time point. It decreased after seeding to values that were comparable at 1 and 7 days of cultivation and consistent with previously published data (8) (Fig. 2D).

**Individual and interactive effects of culture system and culture time on cell viability and metabolic function.** Importantly, both the culture system (perfusion or orbital dish) and time of culture (1.5 h, 1 day, and 7 days) had statistically significant individual effects on the cell survival parameters shown in Fig. 2 (live cell number, cell viability, L/G, glucose consumption). The culture system alone had a significant effect on the levels of LDH in culture medium. For cell viability and L/G, we detected additional interactive effects of culture system and culture time (Table 1).

**Cell cycle.** Cell cycle analysis of the mononucleated cell fraction indicated that there was a proliferative cell compartment in both perfused and dish-grown constructs (Fig. 3). The relative fractions of cells in the G0/G1, S, and G2/M phases determined for the initial cell population (time 0 in Fig. 3, A and B) were maintained throughout the duration of culture in perfused but not dish-grown constructs. Cells isolated from perfused constructs and freshly isolated cardiomyocytes had more cells in the S phase than in the G2/M phase, whereas cells isolated from dish-grown constructs appeared unable to complete the cell cycle and accumulated in the G2/M phase. After 7 days of culture, the percentage of cells in the S phase was significantly higher in perfused than dish-grown constructs (Fig. 3). Statistical analysis demonstrated significant effects of culture system on fractions of cells in the S phase ($P = 0.005$) and G2/M phase ($P = 0.01$) and culture time on the fraction of cells in the G0/G1 phase ($P = 0.018$).

**Tissue architecture.** After 7 days of culture, the overall tissue architecture appeared markedly better for perfused than dish-grown constructs. The 100- to 200-μm-thick peripheral layers of constructs from both groups consisted of tightly packed cells containing cardiac differentiation markers in contrast to construct interiors, which were markedly different (Fig. 4). Medium perfusion maintained high and spatially uniform cell density throughout the construct volume (except in the outer edge regions shielded from fluid flow), whereas molecular diffusion in the interiors of dish-grown constructs supported only a low density of scattered cells.

Sarcomeric α-actin, cardiac troponin I, and sarcomeric tropomyosin were present throughout the perfused construct volume (Fig. 5, A and B). In contrast, dish-grown constructs exhibited spatially nonuniform cell distributions, with most cells expressing contractile proteins located within a 100- to 300-μm-thick surface layer and only a small number of viable differentiated cells in the construct interior (Fig. 5, A and B). Constructs from both groups consisted mainly of mononucleated cells and exhibited a lack of well-established structural alignment of the contractile proteins that was observed in native tissue. The central perfused regions of the constructs (5 mm diameter × 1.5 mm thick) consisted of tightly packed cells containing sarcomeric α-actin, cardiac troponin I, and sarcomeric tropomyosin, similar to neonatal rat ventricles, and distinctly different from the tissue present in the centers of dish-grown constructs (5 mm diameter × 1.5 mm thick), which contained only isolated and poorly differentiated cells (Fig. 5B).

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**Table 1. P values for individual and interactive effects of culture time and culture system on cell viability and metabolism**

<table>
<thead>
<tr>
<th>Culture time</th>
<th>Live Cell Number</th>
<th>Cell Viability</th>
<th>L/G</th>
<th>Glucose Consumption</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture system</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Culture time × system</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.035</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values were determined by two-way ANOVA in conjunction with Tukey’s post hoc test. $P < 0.05$ was considered significant. L/G, lactate-to-glucose ratio; LDH, lactate dehydrogenase; NS, not significant.

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Fig. 3. Fractions of cells in the G0/G1, S, and G2/M phases of the cell cycle. A: perfused constructs. B: dish-grown constructs. At timed intervals, engineered cardiac constructs were digested with collagenase II-dispase, collected cells were permeabilized and labeled with propidium iodide, and DNA was determined using fluorescence-activated cell sorting. Time 0 denotes freshly isolated neonatal cardiac myocytes. Open bars represent the percentage of cells in the G0/G1 phase; light gray bars represent the fraction of cells in the S phase; dark gray bars represent the fraction of cells in G2/M phase. Data are expressed as means ± SE. $P$ values were calculated by two-way ANOVA in conjunction with Tukey’s test for pairwise multiple comparisons ($n = 2–4$ samples/data point). Differences were considered statistically significant if $P < 0.05$. *Significant difference between the fractions of cells within orbital dish group; #significant difference between the corresponding perfused and dish-grown constructs.
Contractile behavior. Spontaneous contractions were observed in some constructs early in culture (dish-grown constructs 2–3 days after seeding) and ceased after ~5 days of cultivation. At the end of cultivation (day 7), spontaneous contractions were not observed in either group of constructs. In response to electrical stimulation (e.g., at 5 V and 60 beats/min), constructs from both groups were reproducibly induced to contract synchronously (see Radisic_video_1 and Radisic_video_2; http://ajpheart.physiology.org/cgi/content/full/00171.2003/DC1). However, in perfused constructs the contraction frequency was constant, whereas in dish-grown constructs the contraction frequency spontaneously increased every 1 to 2 min and the contraction pattern appeared arrhythmic. The ET was significantly lower in perfused than dish-grown constructs, and all construct ETs were significantly higher than those measured for neonatal rat ventricles. There was no significant difference in the MCRs measured for the two groups at suprathreshold stimulus amplitudes (150% and 200% ET; Table 2).

Upon incubation with PA, a gap junction blocker, synchronous construct contractions could not be induced even at 9.9 V. Contractile activity resumed after the washout of PA. The MCRs of perfused constructs and neonatal ventricles were similar to those recorded before PA treatment, suggesting that the effect of PA was completely reversible. However, the MCRs of dish-grown constructs failed to recover their baseline levels after PA washout (Table 2).

DISCUSSION

Tissue-engineered grafts for the repair of impaired myocardium should ideally meet the following requirements: 1) sufficient size and thickness to allow for the repair of infarcted areas or congenital malformations; 2) spatially uniform and physiological density of viable, metabolically active cells (~10⁸ cells/cm³); 3) presence of contractile proteins and other cardiac markers of cell differentiation; 4) functional coupling resulting in synchronous macroscopic contraction in response to stimuli; and 5) mechanical integrity to allow for surgical implantation. Previous studies of engineered cardiac tissue have demonstrated cell coupling and differentiation (24, 36) and mechanical (36) and electrophysiological (3) function. Implantation of engineered constructs has been studied in small animal models (18, 19, 26, 34). One overwhelming limitation of all previous studies, the small thickness of viable and functional engineered tissue (100–200 µm), was attributed to the diffusional transport limitations of oxygen supply (6, 7), and the need to utilize perfusion as a potential means to overcome this limitation has been recognized (34).

Medium perfusion was used in our previous studies to culture cardiac constructs seeded in orbitally mixed dishes (6, 7). Perfusion resulted in spatially uniform cell distributions, but the cell densities of the constructs remained low due to diffusional limitations of oxygen supply during cell seeding (6, 7). In particular, our previous seeding techniques required a 72-h
Differences were considered statistically significant between perfused and dish-grown constructs; ‡ difference between constructs and neonatal rat ventricles; † significant difference before and after PA treatment.

Table 2. Contractile properties of neonatal ventricles, 7-day perfused constructs, and 7-day dish-grown constructs

<table>
<thead>
<tr>
<th></th>
<th>Neatlonal Rat</th>
<th>Perfuased</th>
<th>Dish-Grown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventricle</td>
<td>Construct</td>
<td>(7 days at 500 µm/s)</td>
<td>Construct (7 days at 25 rpm)</td>
</tr>
<tr>
<td>ET, V</td>
<td>Before PA</td>
<td>1.6±0.1</td>
<td>4.5±0.4†</td>
</tr>
<tr>
<td></td>
<td>After PA</td>
<td>1.5±0.1</td>
<td>4.4±0.1‡</td>
</tr>
<tr>
<td>MCR at 150% ET, beats/min</td>
<td>Before PA</td>
<td>413±7</td>
<td>420±30</td>
</tr>
<tr>
<td></td>
<td>After PA</td>
<td>465±15</td>
<td>415±35</td>
</tr>
<tr>
<td>MCR at 200% ET, beats/min</td>
<td>Before PA</td>
<td>427±40</td>
<td>415±45</td>
</tr>
<tr>
<td></td>
<td>After PA</td>
<td>427±58</td>
<td>435±45</td>
</tr>
</tbody>
</table>

Values are means ± SE. Excitation threshold (ET) was measured at a stimulation frequency of 60 beats/min. The maximum capture rate (MCR) was measured at voltages equivalent to 150% and 200% ET. Data were collected before the treatment with palmitoleic acid (before PA) and after the washout of palmitoleic acid (after PA). Constructs could not be induced to contract in the control solution containing PA. P values were calculated by one-way ANOVA in conjunction with Tukey’s test for pair-wise multiple comparisons (n = 2–6). Differences were considered statistically significant if P < 0.05. *Significant difference between constructs and neonatal rat ventricles; ‡: significant difference before and after PA treatment.

Culture system and time also affected in a statistically significant manner the number of cells in the S and G2/M phases of the cell cycle (Fig. 3). Additional interactive effects of culture system and culture time on cell viability and L/G were also detected (Table 1). Notably, perfusion improved all parameters that determine cell survival and function in engineered constructs at all times during the cultivation (Figs. 2 and 3), presumably due to the convective-diffusive transport of oxygen, compared with oxygen diffusion within dish-grown constructs.

Although constructs cultivated in perfusion contained significantly more live cells after 1 and 7 days in culture (Fig. 2A), the live cell numbers decreased with time in both systems. The initial decrease in live cell numbers observed 1.5 h after seeding (Fig. 2A) can be attributed to the insufficient oxygen supply to the cells in the peripheral region shielded from fluid flow in perfused constructs (Fig. 1A, inset) and in the interior of dish-grown constructs. Poor oxygenation of the outer edge of the perfused constructs is consistent with the finding that the volume fraction of the construct that was adequately perfused corresponds roughly to the fraction of surviving cells (live cell number/seeded cell number) after 7 days of cultivation (Fig. 2A). The perfused volume fraction (and thereby the fraction of surviving cells) would increase for thinner silicone gaskets and larger diameter scaffolds. The rather slow decrease of live cell numbers in perfused constructs between 1 and 7 days (Fig. 2A), in conjunction with the maintenance of cell viability (Fig. 2B) and aerobic cell metabolism during the entire cultivation period (L/G ~ 1; Fig. 2C), suggests that mechanisms other than diffusional limitations of oxygen supply are responsible for the decrease in live cell number in perfused constructs. The relatively low cell viability and live cell numbers in dish-grown constructs (Fig. 2A and B) are consistent with the prevalently anaerobic cell metabolism (Fig. 2C) resulting from diffusional transport of oxygen. As a result, a “viable shell” formed at the construct surface within 1.5 h of cell seeding. Approximately constant cell viability in this shell is also consistent with the diffusional limitations of oxygen transport from the construct surface into the interior.

High cell viability is essential for the use of engineered constructs as tissue models for controlled in vitro studies (e.g., cell function, tissue development) as well as for the envisioned in vivo applications. Cell viability was significantly higher in perfused constructs than in orbitally mixed dishes at all time points (Fig. 2B). A transient decrease in cell viability after 1 day of culture in perfusion is consistent with the washout of dead cells from the perfused constructs. If not removed promptly from the tissue construct, cells that die by necrosis swell and burst, releasing their intracellular contents, which can damage surrounding cells (21). In vivo, the intracellular contents of the cells that die by apoptosis are cleared by phagocytosis of apoptotic bodies, the process usually carried out by macrophages. With a lack of efficient clearance, the apoptotic bodies undergo a process called secondary apoptosis and release the lysosomal content into the environment (17, 33). In our in vitro system, there are no efficient cellular means of clearing apoptotic bodies. Even fibroblasts, which have some phagocytic ability, were demonstrated to undergo secondary apoptosis in vitro (27). Importantly, improper removal of apoptotic cells in vivo has been associated with a number of pathological states, e.g., osteoarthritis (22), lupus erythemato-
sus (13), and cystic fibrosis (14, 30, 31). Therefore, the perfusion of medium provides a means for the washout of apoptotic and necrotic cells, thereby minimizing the presence of their intracellular contents within tissue constructs.

As an independent measure of cell damage and death, we monitored the level of LDH in the culture medium in both systems, ensuring that both cells contained within the tissue construct and cells present in culture medium were accounted for. The LDH assay has been used to complement cell viability determined by membrane exclusion dyes [e.g., propidium iodide (29, 32) or calcein AM (23)]. Because LDH is an intracellular enzyme, its presence in the culture medium is indicative of irreversible cell damage and correlates with decreases in cell viability and live cell numbers. Significantly lower levels of LDH in perfused constructs compared with the orbitally mixed dishes at all time points thus indicated that perfusion reduced the level of cell damage. The slow increase in LDH levels (Fig. 2E) in orbital dishes parallels the increase in L/G (Fig. 2C), whereas the constant levels of LDH in perfused constructs parallel the relatively constant L/G, a relationship that has previously been demonstrated for LDH and lactate output (10, 16).

There was no apparent difference in the rate of glucose consumption per unit live cells between dish-grown and perfused constructs, although the live cell numbers differed by a factor of two (Fig. 2D), a result consistent with the fact that anaerobic metabolism yields twice as much lactate per unit glucose as aerobic metabolism. The rate of glucose consumption exhibited a transiently high value 1.5 h after seeding and then decreased to a constant value, consistent with the reported data for glucose consumption in the absence of fatty acids (8).

The maintenance of the relative fractions of cells in the various phases of the cell cycle at levels determined for the initial cell population in perfused but not dish-grown constructs is likely to be due to the better control of cellular environment. Cell cycle analysis revealed a proliferative compartment in both groups of constructs (Fig. 3), indicating that the measured live cell number resulted from a balance of cell death and proliferation in the population of cardiomyocytes and fibroblasts used to seed the scaffolds. The higher number of live cells in perfused than dish-grown constructs (Fig. 2A) is consistent with the higher fraction of cells in the S phase. Importantly, the presence of fibroblasts can improve the mechanical properties of engineered cardiac tissue (36).

The level of structural alignment of contractile proteins, which was subnormal in both groups of engineered constructs, can potentially be improved by utilization of physical forces [e.g., mechanical stimulation (36)]. Therefore, the perfusion system that maintains cell viability, aerobic metabolism, and uniform distribution could be further improved by the addition of defined physical forces. An ideal system would likely include the interstitial medium flow through the construct along with mechanical and/or electrical signals.

The ET for synchronous macroscopic contractions, which was the lowest for neonatal rat ventricles and the highest for constructs cultivated in orbitally mixed dishes, is consistent with observed differences in tissue architecture. In perfused constructs, with regions of densely packed cells distributed throughout the construct volume, ETs were lower than in dish-grown constructs, where densely packed cells were present only at construct surfaces. MCR was measured at 150% and 200% ET to take into account cell regions that were poorly connected to the cell network and may have higher ETs. Comparable MCRs measured at 150% and 200% ET indicate that the cell network was established in both groups without large unconnected regions, at least in the surface region. However, the measured electrical properties are not necessarily a predictor of wave propagation in engineered constructs. Wave propagation studies will be needed to further characterize the spatial homogeneity of functional cell coupling.

As expected, the addition of PA resulted in a cessation of contractile activity, indicating that signal propagation through gap junctions was crucial for synchronous macroscopic contractions. The effect of a gap junction blocker on ET and MCR was completely reversible in neonatal rat ventricles and perfused constructs but not in dish-grown constructs (Table 1), suggesting that the level of organization of the tissue architecture and the robustness of the cell response were different in the two groups.

Tissue constructs cultivated in perfusion may be utilized for in vitro testing to optimize the methods of cultivation and generate constructs suitable for implantation. Although perfused constructs meet most of the requirements necessary for the in vivo implantation (e.g., size, cell uniformity, viability, contractility, and metabolic activity), some requirements are yet to be achieved (e.g., modulation of cell morphology). Several previous studies have shown the feasibility of implanting polymers seeded with cardiac or stem cells (18, 19, 26, 34). Cell survival after implantation likely depends on oxygen supply into the construct interior throughout the period needed for the development of a functional capillary network and construct integration with the host myocardium. We therefore envision the use of constructs with an array of channels that will be implanted parallel to the epicardial surface (to reduce diffusional distances for mass transport and provide space for ingrowth of new blood vessels). To further enhance angiogenesis and vasculogenesis, constructs can be implanted under a membrane containing a vehicle for VEGF release. Together, the use of channeled constructs and transplantation techniques that promote oxygen supply to the cells and the ingrowth of new blood vessels are expected to result in improved cell survival and implant function compared with previously published methods.

In conclusion, interstitial flow of culture medium yielded thick, compact, and contractile cardiac constructs with physi-
ological densities of metabolically active and viable cells containing cardiac markers. The maintenance of oxygen supply to the cells throughout the duration of in vitro cultivation was key for maintaining cell viability and function. These constructs might potentially be used for myocardium repair or studies of normal and pathological tissue function in vitro.

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