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Bioengineering Cardiac Tissue Constructs with Adult Rat Cardiomyocytes

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Abstract

Bioengineering cardiac tissue constructs with adult cardiomyocytes may help treat adult heart defects and injury. In this study, we fabricated cardiac tissue constructs by seeding adult rat cardiomyocytes on a fibrin gel matrix and analyzed the electromechanical properties of the formed cardiac tissue constructs. Adult rat cardiomyocytes were isolated with a collagenase type II buffer using an optimized Langendorff perfusion system. Cardiac tissue constructs were fabricated using either indirect plating with cardiomyocytes that were cultured for one week and dedifferentiated or with freshly isolated cardiomyocytes. The current protocol generated $(3.1 \pm 0.5) \times 10^6$ (n=5 hearts) fresh cardiomyocytes from a single heart. Tissue constructs obtained by both types of plating contracted up to 30 days, and ECG signals and contractile twitch forces were detected. The constructs bioengineered by indirect plating of dedifferentiated cardiomyocytes produced an ECG R wave amplitude of $15.1 \pm 5.2 \mu\text{V}$ (n=7 constructs), a twitch force of 70–110 μN , and a spontaneous contraction rate of about 390 bpm. The constructs bioengineered by direct plating of fresh cardiomyocytes generated an ECG R wave amplitude of $6.3 \pm 2.5 \mu\text{V}$ (n=8 constructs), a twitch force of 40–60 μN , and a spontaneous contraction rate of about 230 bpm. This study successfully bioengineered 3D cardiac tissue constructs using primary adult cardiomyocytes.

Keywords

bioengineering; cardiac tissue constructs; adult cardiomyocytes; electromechanical properties

Introduction

Cardiovascular disease is responsible for greater mortality than all cancers combined in the Western world.¹ This epidemic is progressively worsening with an aging population and an increase in obesity.² More than 50% of patients with heart disease do not respond to current pharmacological therapies.³ Myocardial infarction causes irreversible damage to the

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Conflicts of Interest

None

myocardium, which is then replaced with fibrotic tissue since the heart has minimal intrinsic ability to regenerate lost cardiomyocytes (CMs).⁴ Although mechanical ventricular assist devices can bridge hearts in end-stage failure to donor transplants, biocompatibility, thrombogenicity, and risk of infection complicate their clinical application. Heart transplants remain the gold standard treatment option for end-stage heart failure; however, a shortage of donor hearts and the accompanying need for long-term immunosuppression present major limitations. The development of a tissue-engineered integrative platform would offer a therapeutic option that overcomes the limitations associated with current treatment modalities.^{3, 5, 6} Engineered cardiac tissue constructs have tremendous potential to offer alternative treatment modalities in the healing process of large injured areas in hearts by providing a sufficient number of cells and additional tension support to the damaged area.^{7, 8} Functional and mature bioengineered cardiac tissues could also allow screening of cardiotoxic effects of pharmaceutical agents and facilitate the discovery of new therapeutic agents.^{9–11} The first 3D heart tissues were fabricated with embryonic chick heart cells,^{12–16} followed with fetal cardiac cells,^{17–19} and then with primary neonatal cardiac cells.^{20–22} Since CMs can be derived from embryonic stem cells,^{23–26} induced pluripotent stem cells,^{27–29} or even from mesenchymal stem cells,^{30–32} the availability of cells for the generation of large tissue constructs is no longer the limiting factor.

There has, however, been an evident lack of investigation into the use of primary adult CMs as a cell source to bioengineer 3D cardiac tissue constructs. This can be attributed to challenges in the isolation/culture of these cells, along with the inherent difficulty in maintaining the differentiated phenotype of adult CMs over extended culture periods.^{33–36} Coculture of adult human cardiac fibroblasts and/or adult human CMs or treatment with conditioned medium from the coculture of human cardiac fibroblasts and CMs increase the differentiation of BMP2-induced SSEA-1⁺ cardiac progenitors into mature ventricular CMs that expressed actinin arranged in sarcomeric units within a week,³⁷ thus, cardiac tissue constructs fabricated using adult CMs might be used for guiding cardiac stem cell differentiation and maturation. In this study, we compared two plating protocols to bioengineer 3D cardiac tissue constructs using primary adult rat CMs.

Materials and Methods

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Houston, in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, Revised 2011). All materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Isolation of adult CMs

Buffers were prepared as previously reported.³⁵ Buffer A (500 ml) consisted of NaCl 118 mM, KCl 4.8 mM, K₂HPO₄ 1.25 mM, MgSO₄ 1.25 mM, HEPES 25 mM and Glucose 11 mM, adjusted to pH 7.4 using 10 N NaOH, and filtered through 0.20 µm filter. Buffer B (120 ml) was made by adding CaCl₂ 1.5 mM to sterile buffer A. Enzyme buffer (50 ml) was made by adding butanedione monoxime 5 mM, carnitine 2 mM, taurine 5 mM, glutamic acid 2 mM and 2.5 µM CaCl₂ to 50 ml of buffer A, and before use 0.05% collagenase type II

(Worthington Biochemical Corporation) and 0.02% protease from streptomyces griseus type XIV were added, and the buffer was filtered through a 0.20 μ M filter. Rinsing buffer 1, 2 and 3 (each 50 ml) were made by adding 1.0 mM, 1.25 mM and 1.5 mM CaCl_2 each, and butanedione monoxime 5 mM, taurine 5 mM, BAS 0.1% to 50 ml of buffer A, filtered through a 0.20 μ M filter. Culture media consisted of 20 mM creatine, 2 mg/ml aminocaproic acid, 1% antibiotics-antimycotic containing penicillin, streptomycin, and amphotericin B, 5% FBS, 5% horse serum, 40 ng/ml hydrocortisone, 100 ng/ml insulin and 89% M199 (Life Technologies, Grand Island, NY, USA), filtered through a 0.20 μ M filter.

Fresh adult CMs were isolated from 40 hearts of male Sprague-Dawley rats weighing 300 ± 10 g by an optimized Langendorff perfusion system (Figure 1A), following a protocol previously described.³⁵ Briefly, all parts of the perfusion system were sterilized with 80% ethanol and then irradiated under UV overnight before the system was assembled for use. The perfusion system was rinsed with buffer A, and then 50 ml of buffer A was loaded into buffer A reservoir, and 50 ml of buffer B into the buffer B reservoir. The animals were anesthetized with 2% isoflurane. The hearts were excised and immersed in 20 ml of ice-cold buffer B, and then immediately mounted on an aorta cannula with 4-0 braided silk suture (Figure 1B and C) and perfused with buffer B within 30 seconds. After perfusion with buffer B for 2–3 min, the hearts were primed to buffer A. When buffer A was nearly empty, 50 ml of enzyme buffer was added. During enzyme perfusion, the heart was kept inside closed glassware at 37°C. The enzyme solution was circulated by a peristaltic pump. The above-mentioned procedures were critical for the successful isolation of heart cells (Figure 1D and E). Freshly isolated CMs were allowed to recover in calcium by serially suspending them in rinsing buffers 1 to 3 which contained an increasing concentration of calcium at 1 mM, 1.25 mM and 1.5 mM. Isolated CMs from each heart were counted manually with a Hausser Scientific Hemocytometer (Fisher Scientific), and kept separate from other heart isolations. Based on our preliminary studies (data not shown), we used adult CMs from isolations that obtained greater than 2.50×10^6 cells per heart. Isolations with fewer than this amount resulted in greater variability, and lower cell viability.

Indirect and direct cell plating

For indirect plating of cultured CMs, freshly isolated cells from one heart were diluted with culture media and placed into a 10-cm culture plate coated with 0.3% gelatin at the density of 10,000 CMs/cm² and cultured inside an incubator at 37°C and 5% CO₂. After a 12-hour (or overnight) attachment period, unattached cells were transferred to another gelatin coated plate and cultured for one week. On day 7, the dedifferentiated adult cardiac cells were detached using 0.05% trypsin/2.0 mM EDTA in calcium-free PBS. Cell viability was analyzed by Trypan blue (4%) staining, and CMs and non-CMs were distinguished and counted manually with a Hausser Scientific Hemocytometer by visual inspection. Note that CMs were too big to be characterized by flow cytometry.³⁸ Because adult non-CMs, especially fibroblasts, are much smaller than adult CMs, and we observed that non-CMs dissociated quicker than CMs during trypsinization, we could prepare an appropriate ratio of CMs to non-CMs for plating by mixing different collections of the cell suspension from different plates. As non-CMs (fibroblasts and endothelial cells) possessed a high, and adult CMs possessed a limited proliferation rate, and overpopulation would dramatically affect the

duration of continuous contraction and electromechanical performance.³⁹ Ratios of CMs to non-CMs of 1:1, 1:1.5, 1:2, 1:2.5 and 1:3 were tested in pilot study (data not shown), finally the ratio of CMs to non-CMs of 1:1.5 was determined to be optimal. In this study, each ml of culture media contained a total of 50,000 CMs and 75,000 non-CMs. In indirect plating of cultured CMs protocol, 40 plates were prepared, 20 plates were used for observing continuous contractions and 20 for recording electromechanical properties. For direct plating of fresh CMs, after rod-like cardiac cells with resolved striations from one heart were recovered in calcium in rinsing buffers 1 to 3, they were diluted to a density of 160,000 cells/ml in culture media and cultured inside an incubator at 37°C and 5% CO₂; in this step, there was a certain number of non-CMs that attached to CMs and were unable to distinguish from CMs by visual inspection. Twelve plates were prepared for observing continuous contractions and another 12 plates were used for recording electromechanical properties.

Fabrication of cardiac tissue constructs

Cardiac tissue constructs were fabricated as previously described.³⁹ Briefly, a 35-mm tissue culture plate was coated with 1.5 ml SYLGARD (PDMS, type 184 silicone elastomer). The plate was air dried for 2 weeks and sterilized with 80% ethanol before use. Four minuten pins (Fine Science Tools, Foster City, CA, USA), 0.1 mm diameter, were placed in the culture plate to form a 2 × 2 cm square. The fibrin gel was made by plating 1 ml culture media containing 5 U/ml thrombin, adding 0.5 ml saline containing 5 mg/ml fibrinogen, and mixing well to promote gel formation within 3 min. For indirect plating of dedifferentiated CMs, 125,000 dedifferentiated CMs and 187,500 non-CMs in 2.5 ml of culture media were transferred to the fibrin gel plate. The cells were cultured in the incubator at 37°C and 5% CO₂ with media changes every other day. For direct plating of fresh CMs, 400,000 fresh CMs were diluted in 2.5 ml of culture media, transferred to the fibrin gel plate, and cultured in an incubator at 37°C and 5% CO₂. Pilot studies showed high density of adult CMs plating would cause greater death rate within the first 3 days, which caused acidosis, as evidenced by phenol red pH-sensitive change to yellow in media, and which resulted in reduced cell attachment to the fibrin gel matrix (data not shown). To increase the attachment of live CMs to the matrix, media was titrated to pH 8.0 with 10 N NaOH to neutralize the acidosis caused by the death and degradation of the adult CMs. Half the volume of the culture media was changed with pH 8.0 media every day during the first 4 days, and followed by changes of regular media every other day.

Construct contraction rate and formation

Spontaneous contraction of cultured tissue constructs was observed from day 1 to day 30 after cell plating. The construct growth progress was captured in still photographs and videos, using a camera mounted on an inverted phase-contrast microscope (Nikon Instruments Inc.). The videos were replayed slowly, and the contraction rates manually counted.

Contractile twitch force and electrocardiogram (ECG)

At the time of tissue construct formation, spontaneous and electrically paced twitch forces (10 V, 0.1 s) were measured within a thermostated water bath (37°C) using a high-sensitivity isometric force transducer (MLT0202, ADInstruments, Colorado Springs, CO, USA)

connected to a quad bridge amplifier (FE224, ADInstruments). ECG signals were recorded using a Bio Amp (FE136, ADInstruments) as previously described.³⁹ Data were acquired through a 16-channel PowerLab system (PL3516/P, ADInstruments). The ECG of the tissue construct was detected by inserting a needle cathode (MLA1213, ADInstruments) into the center of the construct and a needle anode in one of the four construct corners. The media immersing the tissue was used as a ground. The contractile twitch force was measured by attaching the force transducer arm to one free-corner of the square construct, while the other three ends of the square construct were held fixed by pins. Pre-tension was adjusted using a micro-manipulator (Radnoti LLC, Monrovia, CA, USA), set at 1000–2000 μN , force during spontaneous contraction was recorded. LabChart (ADInstruments) was used for data analysis. Electrical pacing was performed by attaching stimulating electrodes (MLA0320, ADInstruments) to the edge of the tissue and pacing at frequencies of 0.25 and 1–5 Hz (in 1-unit increments).

Morphological properties

For calculation of twitch force per mm^3 or per g, tissue constructs were cut diagonally after the measurement of contractile twitch forces. The diagonal edge of the triangular block was aligned with the edge of a slide. The cross section and the surface of the triangle block were photographed, and the images were traced using ImageJ 1.47d (Wayne Rashand, National Institute of Health, USA) to measure the area and the thickness, and the volume in mm^3 was calculated. For observation of morphological properties at the formation of tissue constructs, fresh tissue constructs were directly fixed in ice-cold acetone for 10 min; $1.0 \times 1.0 \text{ cm}$ tissue blocks from the center were trimmed, non-specific epitope antigens were blocked and the cell membranes were permeated with 10% goat serum/0.5% Triton X-100 PBS at room temperature for 45 min. Tissue blocks were then incubated in mouse anti- α -actinin (1:200, Sigma, A7811), rabbit anti-collagen type I (1:100, Abcam, ab34710), rabbit anti-laminin (1:150, Abcam, ab11575), rabbit anti-von Willebrand factor (vWF; 1:750, Abcam, ab6994), rabbit anti-connexin 43 (Cx43; 1:100, Abcam, ab11370) and mouse anti-vimentin (1:150, Sigma, C9080) antibodies at room temperature for 2 h. Subsequently, tissue blocks were treated with goat anti-mouse and goat anti-rabbit secondary antibodies (1:400; AlexaFluor 488, 546 and 633, Thermofisher) at room temperature for 1 h. Nuclei were counterstained with 2.5 $\mu\text{g}/\text{ml}$ DAPI for 5 min at room temperature. Fluorescent images were obtained using a Nikon C2+ confocal laser-scanning microscope. For measurement of the volume proportion of myofibrils and extracellular matrix, signal volumes of α -actinin and vimentin were examined within cardiac tissue blocks. Two Z-stack scans from each sample block were acquired, with a signal depth of 28 μm over 33 frames. After determining specific thresholds for α -actinin, vimentin, and nuclei, signal volumes for each sample were measured. The signal volume proportion of α -actinin was expressed as:

$$\alpha - \text{actinin signal volume proportion} = \frac{\alpha - \text{actinin signal volume}}{(\alpha - \text{actinin signal volume} + \text{vimentin signal volume} + \text{nuclear signal volume})} \times 100 \%$$

The proportions obtained from 2 Z-stacks were averaged for each sample.

Statistics

Results are presented as mean $\pm$ standard deviation (SD). Comparisons between two groups were made using the independent samples t-test and comparisons among groups were made using a one-way analysis of variance followed by a Turkey post hoc comparison test. In all tests, differences were considered statistically significant at a value of $p < 0.05$.

Results

Harvest of fresh CMs

In this study, we used a total of 40 hearts for cardiac cell isolations, 20 heart isolations were less than 2.5×10^6 , and were excluded for this study. Reasons for that included: 1) unexpectedly low heart beating rate (< 200 – 250 bpm) after mounting onto the aorta cannula of the Langendorff perfusion system; 2) a delay in the start of heart perfusion due to repeated attempts to mount the heart; 3) the aortic valves were touched or damaged while mounting the heart onto the cannula; 4) technical problems with the perfusion system such as an enzyme buffer that did not reach 37°C . The isolations that strictly followed the current optimized protocol provided $(3.1 \pm 0.5) \times 10^6$ ($n=5$ hearts) rod-like CMs with resolved striations (Figure 1D–E and 2A). Most of the fresh isolated CMs showed spontaneous contractions (**Supplemental movie 1**).

Indirect plating with cultured CMs

Twelve-hour or overnight attachment culture eliminated most non-CMs from the CM suspension and helped to maintain more purified CMs after dedifferentiation. As shown in Figure 2B–D, 2 days after plating, rod-shaped CMs began to alter their shape from oval to round and attached to the flask. The attached cylindrical cells morphed into various shapes and sizes with several pseudopodia-like processes. Most of these irregularly shaped cells resumed spontaneous contractions, but the rate of contraction was not uniform (**Supplemental movie 2**). The number of dedifferentiated CMs harvested from one heart after 7 days of culture was $0.62 \pm 0.15 \times 10^6$ ($n=5$ hearts) as counted manually on a Hausser Scientific Hemocytometer. Figure 2E–H shows representative images of CMs plated on a fibrin gel matrix and in different stages of dedifferentiation. One to two days after plating, some CMs started to spontaneously contract (**Supplemental movie 3**), and on 3–5 days after plating, their contraction synchronized (**Supplemental movie 4**). This synchronization triggered the edge detachment of the fibrin gel and the beginning formation of cardiac tissue constructs (Figure 2I–J). About 50% of cultured tissue plates could form a square shape patch as shown in Figure 2K–L.

Direct plating with fresh CMs

As shown in Figure 3A–D, isolated fresh CMs from one heart plated on fibrin gel plates changed their shape from rod-like to oval and round, and then attached to the fibrin gel matrix. Some of CMs maintained contractions until they attached and dedifferentiated (**Supplemental movie 5**). The dedifferentiated CMs resumed beating and then synchronized to a single contraction at 4–5 days after plating (**Supplemental movie 6**). At 10–13 days after plating, the edge of the fibrin gel began to detach due to the cell contractions. At 13–16

days after plating, the fibrin gel further compacted, and at 16–19 days, about 80% of cultured plates could form square shaped patches as shown in Figure 3G–H.

Tissue construct contraction rate and ECG signal

Both after indirect plating of cultured CMs and direct plating of fresh CMs, the initial non-uniform contractions synchronized into a homogenous rhythmic rate on days 4–5 after plating, and the synchronous contraction sustained until 30 days after plating. As shown in Figure 4A, the maximum contraction rate from indirect plating of cultured CMs was 387 ± 95 bpm ($n=7$ constructs) and occurred on day 10. After that, the contraction rate decreased significantly with time in culture. On day 30, the contraction rate was 130 ± 69 bpm ($n=7$; $p<0.01$ vs day 10). As shown in Figure 4B, the maximum contraction rate from direct plating of fresh CMs was 230 ± 89 bpm ($n=8$ constructs) and occurred on day 5 when all the CMs synchronized to a single contraction; then the contraction rate decreased significantly with time in culture. On day 30, the contraction rate was 134 ± 56 bpm ($n=8$; $p<0.05$ vs day 5). An ECG signal was detected from tissue constructs formed with both plating strategies when they exhibited a visible contraction. The amplitude of the R wave (Figure 4C) from indirect plating of cultured CMs (15.1 ± 5.2 μ V, $n=7$ constructs) was greater ($p<0.01$) than that from direct plating of fresh CMs (6.3 ± 2.5 μ V, $n=8$) (Figure 4D).

Contractile twitch force and pacing response frequency

We defined a spontaneous contraction rate of more than 120 bpm as “high” and a rate of less than 20 bpm as “low” rate. Figure 5A and B showed the representative twitch force plots derived from tissue constructs obtained from both plating strategies. As shown in Figure 5C, the contractile twitch forces measured in constructs formed from indirect plating of cultured CMs that were spontaneously beating at a high rate (72 ± 12 μ N, $n=7$ constructs), or spontaneously beating at a low rate (90 ± 19 μ N, $n=7$), were significantly higher than constructs formed by direct plating of fresh CMs (37 ± 18 μ N, $n=8$ and 60 ± 19 μ N, $n=8$, high and low rate, respectively) ($p<0.05$ or $p<0.01$). When paced at 0.25 Hz and 2 Hz, the tissue constructs formed by indirect plating of cultured CMs had a twitch force of 115 ± 21 μ N ($n=7$) and 73 ± 15 μ N ($n=7$), respectively. These forces were significantly higher than those from direct plating of fresh CMs (63 ± 20 μ N, $n=8$ and 36 ± 21 μ N, $n=8$, at 0.25 Hz and 2 Hz, respectively) ($p<0.01$). Indirect plating also resulted in a greater maximum pacing response frequency (4.1 ± 0.8 Hz, $n=8$) when compared to direct plating (2.6 ± 0.7 Hz, $n=8$) ($p<0.01$) (Figure 5D).

Physical properties

The physical properties of cardiac tissue constructs were measured after the measurement of contractile twitch force. The surface area of the triangular construct block from indirect plating of cultured CMs was 32.1 ± 2.5 mm² ($n=7$ constructs), its thickness was 0.92 ± 0.11 mm ($n=7$), the total tissue volume calculated was 184.9 ± 15.2 mm³ ($n=7$), and its wet weight was 0.49 ± 0.08 g ($n=7$). There were no significant differences in the surface areas, construct thicknesses, and wet weights of the triangular blocks when compared to those from direct plating of fresh CMs ($p>0.05$). Using these measured dimensions, the mean contractile twitch force in cardiac tissue constructs that were spontaneously beating at high and low rate were calculated as 0.39 and 0.48 μ N/mm³ (147 and 182 μ N/g) in indirect

plating of cultured CMs, and 0.21 and 0.33 $\mu\text{N}/\text{mm}^3$ (80 and 124 $\mu\text{N}/\text{g}$) in direct plating of fresh CMs. When paced at 0.25 Hz and 2 Hz, the mean contractile twitch forces were calculated as 0.60 and 0.39 $\mu\text{N}/\text{mm}^3$ (227 and 147 $\mu\text{N}/\text{g}$) in indirect plating of cultured CMs, and 0.35 and 0.20 $\mu\text{N}/\text{mm}^3$ (131 and 76 $\mu\text{N}/\text{g}$) in direct plating of fresh CMs.

The immunofluorescence images (Figure 6A–H) from the cardiac tissue constructs made by indirect plating of cultured CMs and direct plating of fresh CMs showed expression of α -actinin, which is specific to CMs. Positive staining of vimentin and collagen type I indicated that the cardiac fibroblasts maintained the potential for ECM production and support. Positive staining for connexin 43 illustrated that the cardiac tissue constructs has potential for electromechanical coupling, which sustains electrical propagation between cardiac cells. We also observed vWF staining, indicating that the cardiac tissue constructs contained endothelial cells. Volume proportions of the cardiac elements within the constructs were calculated from immunofluorescence staining of α -actinin as an indicator of CMs, vimentin as an indicator of fibroblasts and endothelial cells, and nuclei. The volume proportions of α -actinin, vimentin and nuclei in tissue constructs made by indirect plating of cultured CMs ($n=4$ constructs) were $49\% \pm 14\%$, $22\% \pm 9\%$ and $30\% \pm 6\%$, respectively, and in the tissue constructs made by direct plating of fresh CMs ($n=4$) were $70\% \pm 7\%$ ($p<0.05$ vs indirect), $13\% \pm 2\%$ ($p>0.05$ vs indirect) and $17\% \pm 5\%$ ($p<0.05$ vs indirect), respectively (Figure 7).

Discussion

In this study, two plating strategies, indirect and direct, were assessed to determine appropriate 3D culture conditions for improving adult CM viability, phenotype retention, and functionality. The indirect plating strategy is an attempt to control the initial seeding ratio of CM to non-CM at 1:1.5 onto the fibrin gel, after a 12-hour pre-plating step. The direct plating strategy involves direct plating of fresh CMs onto the fibrin gel after isolation from an adult rat heart.

Approximately 50% of cardiac tissue constructs made by indirect plating with cultured CMs formed square shaped tissue constructs at 12–17 days after plating, with a maximum contraction rate 390 bpm at day 10 after plating, which is equivalent to a normal adult rat heart. For the indirect plating strategy, we plated 125,000 dedifferentiated CMs and 175,000 non-CMs onto one 35 mm culture plate containing a fibrin gel. Our previous study, in which we used neonatal cardiac cells to fabricate a cardiac tissue patch, found that patches obtained from $5\text{--}6 \times 10^6$ cells could contract at 400 bpm, and their tissue thickness was about 21 μm measured from Masson's trichrome staining.³⁹ From this data, we inferred that the number of CMs and non-CMs plated in the 35 mm plates in the current tissue constructs formed from indirect plating reached the maximum number possible for this type of plating. Approximately 80% of cardiac tissue constructs made by the direct plating with fresh CMs formed square shaped tissue constructs at 10–19 days after plating, with a maximum contraction rate of only 230 bpm on day 5. This was likely due to a reduced number of beating CMs as a result of the plating method and media changes. We observed that about 75% of plated adult rat CMs became swollen and eventually degraded and were discarded during media changes. Thus, additional experiments are needed to identify ways by which the rate of attachment and dedifferentiation after plating of fresh isolated adult CMs can be

improved. In the current study, the culture media contained 5% FBS and 5% horse serum plus 40 ng/ml hydrocortisone and 100 ng/ml insulin. Our previous experiments showed that cardiac tissue patches were able to form 2–6 days after plating 1–6 million of neonatal cardiac cells onto a fibrin gel matrix in 35 mm culture plates with culture media containing 10% FBS and 5% horse serum.³⁹ In the current study, the contractions of tissue constructs from both types of plating lasted more than 30 days (Figure 4) (**Supplemental movies 7 and 8**). Thus, the initial plating density, proportion of CMs and non-CMs, the proliferation rate of cardiac cells, type and concentration of chemical/biological additives in the culture media may all impact contraction rate and time.

Electromechanical properties, such as the duration of continuous contraction, the strength of contractile twitch force and the amplitude of ECG QRS complex, largely relies on the appropriate proportion of CMs to ECM, the thickness of the tissue and the actual dedifferentiated CMs that generate cardiac vector.^{27, 39} The current cardiac tissue constructs bioengineered from indirect plating of cultured CMs generated contractile twitch force in the range of 70–110 μN and ECG R wave amplitude in the range of 10–20 μV ; constructs from direct plating of fresh CMs showed contractile twitch forces in the range of 40–60 μN and ECG R wave amplitude in the range of 4–9 μV ; and the constructs from both seeding strategies could contract for 30 days. By dividing block volume or wet weight, we calculated the contractile twitch force in the range of 0.38–0.59 $\mu\text{N}/\text{mm}^3$ or 143–224 $\mu\text{N}/\text{g}$ tissue for indirect plating of dedifferentiated CMs and 0.22–0.32 $\mu\text{N}/\text{mm}^3$ or 82–122 $\mu\text{N}/\text{g}$ tissue for direct plating of fresh CMs. Moreover, the proportion volumes of myofibrils and nuclei from indirect plating were different from those from direct plating. In human, left ventricular mass affects both ECG QRS amplitude and duration in the precordial lead V5 or V6; within normal range, the thicker the left ventricular wall the higher the R wave amplitude.⁴⁰ In this study, the strength of twitch force was much greater and the amplitude of ECG R wave was much higher from indirect plating constructs than those from direct plating constructs ($p < 0.01$), but there were no significant differences in the thickness and the wet weight of constructs in both plating methods. Taken that dedifferentiated adult CMs have a limited proliferation rate,^{41, 42} 125,000 dedifferentiated CMs were used in the indirect plating plates and 400,000 fresh CMs, in which about 75% died, were used in the direct plating plates. When twitch force and ECG R wave were measured, the number of actual dedifferentiated CMs in one indirect plating construct was around 127,550 per gram mass [$125,000 \div (2 \times 0.49)$], and that in one direct plating construct was around 102,040 per gram mass [$400,000 \times 25\% \div (2 \times 0.49)$]. Figure 6E–H and Figure 7B demonstrated that there was a certain number of undifferentiated CMs (arrows) in the direct plating constructs which did not help generate twitch force and ECG R wave. Thus, the different number of actual dedifferentiated CMs could help explain the difference of twitch force and ECG R wave amplitude in these two plating protocols.

Our previous publication showed that cardiac constructs³⁹ and artificial heart⁴³ bioengineered with neonatal cardiac cells could contract for one week. The reason they terminated contraction was the overpopulation of non-CMs (cardiac fibroblasts and endothelial cells) as the non-CMs possessed a high proliferation rate and CMs possessed a limited proliferation rate. In the pilot study, tissues bioengineered with 1:3 of

dedifferentiated CMs to non-CMs would terminate contraction within a few days and could form square shape constructs but no twitch forces and ECG signal could be detected; however, when the ratio of dedifferentiated CMs to non-CMs was set at 1:1 or less, the tissues could contract more than 30 days, but the rate of square shape construct formation was much lower than those of tissues seeding with the 1:1.5 of dedifferentiated CMs to non-CMs. Thus the 1:1.5 ratio of dedifferentiated CMs to non-CMs was selected for the current study.

Conclusions

This study successfully bioengineered a 3D functional cardiac tissue construct using adult CMs. A bioengineered 3D construct composed of adult CMs may be used to aid in the development of clinically applicable tissue engineered heart constructs, for example, autologous tissue constructs for the treatment of myocardial infarction in humans can obviously not be prepared from fetal or neonatal CMs. In this study, the constructs fabricated using dedifferentiated CMs can beat as fast as a normal rat heart. For the indirect plating of cultured CMs, new experiments are needed to quantify the impact of cell density on the strength of the twitch force; and for the direct plating of fresh CMs, new tests are required to find ways to improve the rate of attachment and dedifferentiation of fresh adult CMs.

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Figure 1.

Isolation of adult rat CMs. **A**, Langendorff perfusion system set-up for adult CM isolation; arrows indicate using a pre-warmed glassware and a glass bottle to keep enzyme buffer and the heart at 37°C. **B**, Dissecting the aortic trunk close to the aortic arc; white line shows trimming the aorta before mounting; **C**, Mount the heart above the aortic valves; arrow shows location of aortic valves. **D**, Appearance of heart after a successful enzyme digestion. **E**, Cross-section of a successfully digested heart.

Figure 2.

Formation of cardiac tissue constructs with indirect plating of cultured CMs on fibrin gel matrix. **A–D**, Dedifferentiation of CMs. **A**, Fresh CMs; white arrows indicated live and rod-like CMs, and white arrow heads demonstrated dead and degraded CMs; **B**, Day 2 after plating; **C**, Day 4 after plating; **D**, Day 6 after plating; white arrows indicated live CMs in the process of dedifferentiation, white arrow heads demonstrated dead and degraded CM, and green arrows showed CMs which were dividing. **E–H**, Culturing of dedifferentiated CMs on fibrin gel matrix; **E**, Day 1 after plating; **F**, Day 5 after plating; **G**, Day 9 after plating; **H**, Day 13 after plating; white arrows indicated differentiated cardiac cells, white arrow heads demonstrated dead cardiac cells, and green arrows showed CMs which were dividing. **I–L**, Formation of cardiac tissue constructs; **I**, Days 12–13 after plating; **J**, Days 13–15 after plating; **K**, Days 15–17 after plating; **L**, Days 17–19 after plating.

Figure 3.

Formation of cardiac tissue constructs with direct plating of fresh CMs on fibrin gel matrix.

A, Fresh CMs; white arrows indicated live and rod-like CMs, and white arrow heads demonstrated dead and degraded CMs; **B**, Day 1 after plating; **C**, Day 7 after plating; **D**, Day 14 after plating; white arrows indicated CMs which were changing shape and dedifferentiating, and white arrow heads demonstrate dead cells. **E–H**, Formation of heart tissue constructs. **E**, Days 10–13 after plating; **F**, Days 13–16 after plating; **G**, Days 16–19 after plating; **H**, Days 19–22 after plating.

Figure 4. Contraction rate and ECG signals. **A**, Contraction rate of indirect plating with cultured CMs; **B**, Contraction rate of direct plating with fresh CMs; **C**, ECG of indirect plating. **D**, ECG of direct plating. Values are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

Figure 5.

Contractile twitch forces. **A**, Representative twitch force plots of indirect plating with cultured CMs; **B**, Representative twitch force plots of direct plating with fresh CMs; **C**, Graph of twitch forces from both indirect and direct plating; **D**, Graph of pacing response frequency. Values are mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$.

Figure 6. Immunofluorescence staining. **A–D**, Representative images showing physical properties from indirect plating with cultured CMs; **E–H**, Representative images showing physical properties from direct plating with fresh CMs, arrows indicate undifferentiated CMs.

Figure 7. Proportion volume of physical muscle elements within formed tissue constructs. **A**, The representative image of indirect plating with cultured CMs; **B**, The representative image of direct plating with fresh CMs, arrows indicate undifferentiated CMs; **C**, Graph showing proportion volumes of muscle elements from both plating. Values are mean $\pm$ SD. * $p < 0.05$.