Myocyte-Depleted Engineered Cardiac Tissues Support Therapeutic Potential of Mesenchymal Stem Cells

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The therapeutic potential of mesenchymal stem cells (MSCs) for restoring cardiac function after cardiomyocyte loss remains controversial. Engineered cardiac tissues (ECTs) offer a simplified three-dimensional *in vitro* model system to evaluate stem cell therapies. We hypothesized that contractile properties of dysfunctional ECTs would be enhanced by MSC treatment. ECTs were created from neonatal rat cardiomyocytes with and without bone marrow-derived adult rat MSCs in a type-I collagen and Matrigel scaffold using custom elastomer molds with integrated cantilever force sensors. Three experimental groups included the following: (1) baseline condition ECT consisting only of myocytes, (2) 50% myocyte-depleted ECT, modeling a dysfunctional state, and (3) 50% myocyte-depleted ECT plus 10% MSC, modeling dysfunctional myocardium with intervention. Developed stress (DS) and pacing threshold voltage (VT) were measured using 2-Hz field stimulation at 37°C on culture days 5, 10, 15, and 20. By day 5, DS of myocyte-depleted ECTs was significantly lower than baseline, and VT was elevated. In MSC-supplemented ECTs, DS and VT were significantly better than myocyte-depleted values, approaching baseline ECTs. Findings were similar through culture day 15, but lost significance at day 20. Trends in DS were partly explained by changes in the cell number and alignment with time. Thus, supplementing myocyte-depleted ECTs with MSCs transiently improved contractile function and compensated for a 50% loss of cardiomyocytes, mimicking recent animal studies and clinical trials and supporting the potential of MSCs for myocardial therapy.

Introduction

EACH YEAR, AN ESTIMATED 785,000 Americans will suffer a myocardial infarction (MI), in which a region of heart muscle dies due to insufficient oxygenation, resulting in roughly one death every minute.¹ This alarming mortality rate reflects the fact that adult myocardium does not allow significant regeneration,² resulting in a loss of contractile function that leads to heart failure in 21% of men and 30% of women within 6 years in those that survive MI.³ Therefore, the development of an effective therapy to repair and regenerate damaged myocardium is highly desired.

One promising approach to restoring contractile function is the delivery of cells to the injured myocardium. In particular, mesenchymal stem cells (MSCs) have demonstrated great potential for cardiac repair,⁴ although the biological mechanisms underlying such cell therapies remain unclear.⁵ This at least partly reflects a gap in the available biological assays, which leap from traditional

hard and flat culture surfaces (e.g., Petri dishes) to animal models and man. The development of a biomimetic three-dimensional (3D) *in vitro* cardiac culture system may facilitate the clinical translation of stem cell therapies for heart disease.

Engineered cardiac tissues (ECTs) are surrogate tissue constructs created *in vitro* by placing isolated cardiomyocytes into a natural or synthetic scaffold material. Such ECTs have been shown to mimic myocardial structure and reproduce key aspects of natural cardiac function. Although a long-term goal of cardiac tissue engineering is to create a functional myocardial patch for surgical implantation, ECTs are also useful as *in vitro* models for biomedical research.⁶ The geometry, structure, and cellular composition of ECTs can be experimentally controlled. Moreover, 3D ECTs are more physiologic than standard two-dimensional culture and their long-term viability exceeds that of isolated natural myocardium.⁷ These characteristics make ECT a potentially powerful experimental tool for studying cell-based therapies such

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as MSCs. However, the effect of co-culturing MSCs in ECTs has never been investigated.

In this study, we describe a culture system for creating and noninvasively monitoring the contractile function of rat cell-derived ECTs. The system was used to examine myocyte-depleted ECT as an *in vitro* model of myocardial dysfunction, and to test the hypothesis that supplementing the impaired ECTs with rat MSCs would significantly augment their contractile properties.

Materials and Methods

All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health.⁸ Animal protocols were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

MSC isolation

Bone marrow-derived MSCs were isolated and expanded from adult female Sprague-Dawley rats (Taconic Farms, Germantown, NY) at least 2 months postpartum by modifying an established protocol.⁹ A total of six isolations, using two rats each, were used for this study. Immediately after euthanasia by pentobarbital overdose, the femoral and tibial bones were dissected, removing all muscle layers and epiphyses. The stripped bones were flushed with the warm Hanks Balanced Salt Solution (HBSS) (GIBCO, Carlsbad, CA), and the extruded bone marrow was filtered through a 100- μ m strainer, and the collected sample (30 mL HBSS + bone marrow whole blood) was layered carefully onto 15 mL of warm Ficoll-Paque (GE, Piscataway, NJ). After density centrifugation (300 g for 20 min at room temperature), the resulting pinkish mononuclear cell layer was retrieved and resuspended in cold HBSS (20 mL); after a second centrifugation at 500 g for 10 min, the cell pellet was resuspended in the Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) 1 g/L glucose media containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin. The cells were plated and expanded in culture to passage 4 to yield a population rich in undifferentiated MSCs (see Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/tea), as confirmed using immunocytochemistry by staining for vimentin, a marker of undifferentiated MSCs. The flow cytometry analysis (BD LSR II; BD Biosciences, San Jose, CA) was used to establish the phenotypic characteristics of the cell population using the MSC marker CD90 and the hematopoietic marker CD45. The multilineage differentiation characteristic of MSC was induced using commercial differentiation media following manufacturer's protocols (Invitrogen, Carlsbad, CA) into adipogenic, osteogenic, and chondrogenic lineages, as corroborated by Oil Red O, Alizarin red, and Alcian blue stains, respectively. For the creation of ECT, undifferentiated MSCs (passage 3 or 4) were switched to the DMEM with 4.5 g/L glucose 1 week before harvesting as preparation for the transition to ECT culture conditions.

Neonatal rat cardiomyocyte isolation

Neonatal rat cardiomyocytes (NRCMs) were isolated from ventricles of 1–2-day-old Sprague-Dawley rats (Taconic Farms, Germantown, NY) using a commercial myocyte isolation kit (Worthington, Lakewood, NJ). A total of six isolations using two litters each were used in this study. Following manufacturer's instructions, the myocardial tissue was treated with Worthington Trypsin in HBSS for 16 h followed by 45 min digestion with collagenase. After 10-min centrifugation at 100 g, the cell pellet was resuspended in the NRCM culture medium (DMEM 4.5 g/L glucose, with 10% newborn bovine serum (Hyclone, Rockford, IL), 1% penicillin/streptomycin, and 0.2% amphotericin B. After a preplating time of 35 min to separate neonatal rat cardiac fibroblasts, the nonadherent cells were again suspended in media, and the cell pellet was treated with the red blood cell lysis buffer (RCLB) (EBioscience, San Diego, CA) (3 mL media + 20 mL RCLB) and spun for 10 min at 100 g. Eliminating erythrocytes aided in providing an accurate cell count and a cardiomyocyte-rich population, as confirmed by the flow cytometry analysis using the myocyte marker sarcomeric α -actinin and the fibroblast marker vimentin. The resulting cell pellet was used immediately for creation of ECT.

Polydimethylsiloxane culture molds with integrated cantilever force sensors

ECT culture molds with integrated force sensing posts were fabricated from custom delrin masters using soft lithography with polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning, Midland, MI). This PDMS device has a modular design with components that fit together for creating the ECT, including inserts that provide a rectangular well (13 $\times$ 2 $\times$ 2 mm) having a vertical PDMS post (3.5 $\times$ 0.5 mm) near each end of the well. The posts provide anchors during tissue culture to resist axial gel compaction and induce cell alignment. Similar to recently published devices,^{6,10} the flexible PDMS posts also serve as integrated force sensors that deflect as the tissue beats, allowing noninvasive monitoring of ECT contractile function. The tissue-generated contractile force can be determined from optically measured deflection of the cantilever posts using the classical beam theory as described below.

Creating ECTs

Three different conditions of ECTs were used as experimental groups, varying the cell composition and concentration: (1) baseline condition (15 $\times$ 10⁶ NRCM/mL; *n* = 11), (2) myocyte-depleted (7.5 $\times$ 10⁶ NRCM/mL; *n* = 12), and (3) myocyte-depleted supplemented with MSC (7.5 $\times$ 10⁶ NRCM/mL + 0.75 $\times$ 10⁶ MSC/mL; *n* = 13). Following our established protocols,¹¹ ECTs were created by combining the desired cell concentration with a mix of bovine type-I collagen (GIBCO) and Matrigel (BD Biosciences) and pipetted into the custom PDMS elastomer culture molds. Briefly, an ice-cold sterile collagen solution (1:1:8 mixture of HEPES: 10 $\times$ minimum essential medium (MEM): collagen) was prepared and combined with Matrigel and the desired cell suspension in a 8:1:1 ratio, yielding final concentrations of 2.0 mg/mL collagen type-I and 0.9 mg/mL Matrigel. Approximately 100 μ L of this matrix/cell suspension was pipetted into the PDMS elastomer well that was assembled and precoated with 2% bovine serum albumin

(Sigma) for 1 h at 37°C to minimize cell and collagen adhesion (Fig. 1A). The solution was incubated for 2 h at 37°C and 5% CO₂ to initiate polymerization of the gel in the mold before adding the additional NRCM culture medium. Detachable inserts (Fig. 1B) were removed after 48 h of incubation to visualize spontaneous beating of ECT. Long-term viability was achieved by maintaining the tissues at 37°C and 5% CO₂, immersed in culture media with daily half-volume changes. Vertical posts near each end of the well serve as anchors to guide gel compaction and direct tissue self-assembly into a thin cylindrical engineered cardiac muscle (Fig. 1C).

Monitoring contractile function of ECTs

At four different time points (5, 10, 15, and 20 days after ECT creation), the contractile function was measured utilizing a custom setup. For monitoring ECT active contractile force, the PDMS device holding the tissue was moved to a heated stage placed on a vibration isolation table (NuAire, Plymouth, MN) under a dissecting microscope (SZ40; Olympus, Center Valley, PA) set up in a sterile biological hood (NuAire). A GRASS S88× stimulator (Astro-Med, West Warwick, RI) was programmed to pace the tissues by field stimulation with two carbon rod electrodes spaced 22 mm apart, using a 12-V pulse wave (545 mV/mm) of 5-ms duration at prescribed frequencies from 1–14 Hz, with 1-Hz increments after 500 twitches at each frequency. Using a high-speed digital CMOS camera (PL-B741; PixelLink, Ottawa, ON) attached to the microscope, the moving tip of each flexible post was captured at a rate of 90 frames per second. A custom LabVIEW software program (National Instruments, Austin, TX) was used to track the centroid of the moving post tip in real time during live imaging. The height of the tissue on the post, a , was measured optically by acquiring a lateral view of the tissue at a focal plane that allowed visualization of both posts and tissue, with the aid of a 20-mm right-angle mirror (Edmund Optics, Barrington, NJ). The average Young's modulus of the post was calculated to be 1.33 MPa from calibration tests with a high-sensitivity

force transducer (Scientific Instruments, Heidelberg, Germany). For each tissue, the cross-sectional area (CSA) was measured from a top view using the dissecting microscope and assuming cylindrical geometry of the tissue. The threshold voltage (VT) required for electrical pacing at 2 Hz was measured by incrementally increasing the stimulation voltage until the measured beating frequency of the tissue matched the prescribed pacing frequency.

Data analysis

An analysis of amplitude and frequency of post deflection was performed offline using custom MATLAB routines (MathWorks, Natick, MA). For each contraction, the post position as a function of time was documented by analyzing the change in distance between the post centroids. Each maximum and minimum position was found by polynomial interpolation of the raw data, and the post deflection, δ , was found from the difference (max – min) and averaged for each test condition. This deflection was used to compute the developed force, F , based on the principle shown in Figure 2A and equation shown in Figure 2B, with E , L , and R representing the Young's modulus, length, and radius of the PDMS posts, respectively. This developed force was divided by the average CSA of the tissue sample to yield developed stress (DS). The developed force was also divided by the number of myocytes seeded into each ECT to estimate the developed force per myocyte. To validate whether the tissue was responding to the electrical stimulus at the same frequency as the pacing frequency (i.e., capturing), a fast Fourier transform (FFT) was performed on the post deflection versus time data. If the frequency of the largest peak in the FFT power spectrum matched the pacing frequency, then the ECT was considered to be captured.

Histological analysis

ECTs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored at 4°C until processing.

FIG. 1. Engineered cardiac tissue (ECT) creation. **(A)** Diagram of cell–matrix mixture pipetted into a polydimethylsiloxane (PDMS) mold; gel formation initiates within first 2 h, leading to compaction and self-assembly of cylindrical ECTs anchored by end posts. **(B)** Image of a custom PDMS mold with removable inserts. Top panel: PDMS mold with inserts in place creating a defined 2×2×13-mm rectangular well with posts near each end. Scale bar=10 mm. Lower panel: PDMS mold disassembled, with inserts on the side. **(C)** Image of ECT. Top panel: Top view of ECT attached to end posts. Scale bar=1 mm. Lower panel: Side view of ECT while contracting and inducing inward deflection of the posts.

FIG. 2. Analysis of the post deflection based on the elastic beam theory, allowing calculation of the tissue force applied at some distance from the tip of the post where deflection is measured. **(A)** Schematic of the flexible post analysis following the elastic beam bending equation. F is tissue contraction force; E , R , L represent Young's modulus, radius, and length of the PDMS posts, respectively; a is the height of the tissue on the post; δ is measured tip deflection. **(B)** Representative tracing of ECT contraction during field stimulation at 2 Hz. Each spike represents post deflection, δ , during a tissue twitch. **(C)** Detected beating frequency of representative ECT (using fast Fourier transform analysis of post deflection) versus prescribed pacing frequency. The dotted line indicates a slope of unity, verifying measured beating frequency is equivalent to prescribed pacing frequency until the loss of capture at 14 Hz. **(D)** Corresponding force–frequency relationship shows a decreasing trend over the capture range from 1 to 13 Hz.

Fixed ECTs were embedded in Tissue-Tek OCT Compound (Sakura, Torrance, CA), frozen, and cut into 10- μ m thick sections with a Microm HM560 cryostat (Microm International, Walldorf, Germany) for fluorescent analysis, or embedded in paraffin and cut into 4- μ m thick sections for hematoxylin and eosin (H&E) staining. For immunofluorescence, slides were incubated with ice cold acetone for 20 min, rinsed with PBS (5 min $\times$ 3), and blocked using 10% goat serum for 1 h, followed by incubation with a primary antibody overnight, using mouse anti- α -actinin (sarcomeric) (Sigma A7811; 1:800) and rabbit anti-connexin-43 (Sigma C6219; 1:400) and then with the secondary antibody for 1 h using Alexa Fluor 594 goat anti-mouse IgG (Life Technologies, Carlsbad, CA; A11032, 1:800) and Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies; A11008, 1:800). Nuclei were counterstained using a mounting medium containing 4',6 diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images were obtained using a laser scanning confocal microscope (Leica TCS SP5 DMI; Leica Microsystems, Buffalo Grove, IL) for fluorescence, or an inverted light microscope (Olympus IX71) with a digital color camera (Olympus DP72) for histology.

Characterization of tissue architecture

Cell number, distribution, and orientation were analyzed using one representative ECT from each of the three test groups fixed at 5-, 10-, 15-, and 20-day time points. Frozen

samples were sectioned as above (10- μ m thickness), permeabilized with 0.1% triton X, and stained for filamentous actin with rhodamine-phalloidin (Invitrogen, Eugene, OR) as well as the DAPI nuclear stain. Confocal images were acquired with a 20 $\times$ objective using an automated tiling algorithm within Leica software to obtain whole-width ECT images; this was performed at three locations (left, middle, and right) along the length of each representative tissue. While descriptive statistics revealed the variations within each measured tissue, inferential statistics were not appropriate without multiple independent samples from each group.

Cell number and distribution. The number of cells in each DAPI-stained ECT section was determined using ImageJ software (NIH, <http://rsbweb.nih.gov/ij/>) with the free ITCN automated nuclei counter plug-in (Center for Bio-Image Informatics, University of California at Santa Barbara, www.bioimage.ucsb.edu/automatic-nuclei-counter-plug-in-for-imagej). For each image, a rectangular region of interest (ROI) was defined to extend 500 μ m along the long axis of the ECT and to span the full width of the tissue. The total number of DAPI-stained nuclei in this ROI was used for the cell number at that location. The ROI was then split into three equal adjacent zones (edge, center, and edge), and the number of nuclei in each zone was counted to provide a distribution of cells across the tissue width at that given location. Repeating this for each of the three locations (left,

middle, and right) provided a mean and standard deviation ($n=3$) for each zone of each tissue, which were then compared for the three test groups and the four time points.

Cell alignment. Using the same tissue sections described above, cell orientation was measured in images of ECT stained with rhodamine-phalloidin; ECTs from 48-h and 72-h time points were also evaluated. These were analyzed using MatFiber,¹² a MATLAB script developed to quantify image alignment based on a gradient detection algorithm applied sequentially to adjacent square subregions of the image to determine the predominate orientation of features in each (60×60-pixel) subregion, typically yielding 200 measurements or more per image. The resulting cell orientation data were analyzed using circular statistics to determine the mean vector length, r , which ranges from $r=0$ for a random distribution to $r=1$ for a perfectly aligned sample. As described above, three different locations (left, middle, and right) along the length of each representative tissue sample were combined and analyzed for comparison of cell alignment across time for each group of ECTs.

Statistical analysis

Data were represented by mean and standard deviation values. Differences between groups were evaluated at each time point based on the one-way analysis of variance, with the Scheffe's *post-hoc* test for multiple pairwise comparisons, using SPSS statistics software (IBM Corp, Somers, NY). A p -value <0.05 was considered statistically significant.

Results

Cell characterization

The mesenchyme lineage of MSCs used for ECT creation was supported by immunofluorescence with positive expression for vimentin (see Supplementary Fig. S1B). In addition, the capability to give rise to MSC types (adipogenic, osteogenic, and chondrogenic) was corroborated after inducing differentiation of MSCs into each of these three lineages using Oil Red O, Alizarin red, and Alcian blue stains, respectively (see Supplementary Fig. S1C). Phenotypic characterization of the cells used for ECT creation was determined by the flow cytometry analysis. The MSC cell population was positive for CD90 (99.9%) and negative for the hematopoietic marker CD45 (see Supplementary Fig. S2A). The cells obtained from the NRCM isolation, after 35 min of preplating, consisted of a cardiomyocyte-rich population, with a higher percentage positive for sarcomeric α -actinin (83.2%), and a lower proportion of cells expressing the fibroblast marker, vimentin (26.2%) (Supplementary Fig. S2B).

Characterization of ECT

All ECTs compacted and began to beat spontaneously at ~ 2 Hz between days 3 and 5 in culture. The tissues were cylindrical in shape with a length of ~ 10 mm between endposts; starting from an initial CSA of 4.0 mm^2 defined by the PDMS mold, they compacted down through 20 days in culture to $0.24 \pm 0.11 \text{ mm}^2$ for the baseline group. ECTs consistently responded to pacing by field stimulation (see Supplementary Video S1) to frequencies above 10 Hz, and loss of capture was clearly identified using the FFT analysis (Fig.

2C). A negative force–frequency relationship was observed (Fig. 2D), similar to reports for natural rat myocardium¹³ and other engineered heart tissue created from NRCMs.¹⁴

Immunofluorescence confocal microscopy demonstrated cardiomyocytes that stained positive for sarcomeric α -actinin, with organized sarcomeres readily visible (Fig. 3). The elongated cardiomyocytes were preferentially aligned with the ECT longitudinal axis (i.e., from post to post) along which the ECT contractile force was measured. Positive expression for the gap junction protein connexin 43 was also observed (Fig. 3), exhibiting a punctate distribution that often localized to cell boundaries, but did not appear to form distinct intercalated discs.

H&E stain indicated that tissue integrity was maintained through culture day 20 for all three groups of ECTs (Fig. 4A). The number of DAPI-stained cell nuclei appeared relatively constant over time for myocyte-depleted and MSC-supplemented ECTs (Fig. 4B, C), whereas the baseline ECT exhibited a notable decrease in the number of nuclei from day 5 to day 15. The cell distribution data suggested that these time-dependent trends were similar in all three zones through the tissue thickness (Fig. 4D). All ECT groups tended to have more cells concentrated at the edges of the tissue compared to the central zone, particularly at the later time points, reflecting preferential collagen compaction at the tissue edges due to long-term remodeling processes.

Cell alignment was observed to increase over time in culture as shown in Figure 5. For all groups, the mean vector length increased from $r \sim 0.1$ (i.e., nearly random) at 48 h and 72 h, to $r \sim 0.9$ (i.e., strongly aligned) at 20 days (Fig. 5D). The cell alignment process appeared faster and stronger in the MSC-supplemented group as compared to the myocyte-depleted and baseline ECTs.

Developed stress

As shown in Figure 6, DS (max–min force/area) for the baseline group during pacing at 2 Hz was 56 ± 28 , 143 ± 82 , 181 ± 10 , and 221 ± 25 Pa on days 5, 10, 15, and 20, respectively, indicating an improved function with time in culture. At days 5 through 15, myocyte-depleted tissues showed significantly lower DS compared with the baseline ECT group ($p < 0.02$), with values of 12.0 ± 6.9 , 27.1 ± 7.6 , 61.3 ± 38.5 , and 174 ± 154 Pa at corresponding time points. MSC supplementation of myocyte-depleted ECT in a ratio of 10:1 NRCM:MSC resulted in significantly increased DS at days 5 through 15 compared to the myocyte-depleted group ($p < 0.01$), with average values of 52.5 ± 25.7 , 257 ± 124 , 207 ± 131 , and 325 ± 65.8 Pa, comparable to the baseline group containing twice as many myocytes (Fig. 6).

Cross-sectional area

ECT CSA also varied between groups and tended to decrease with time in culture as a result of cell-dependent collagen compaction (Fig. 7). Baseline ECT had an average CSA of 0.33 ± 0.10 , 0.29 ± 0.09 , 0.25 ± 0.11 , and $0.24 \pm 0.11 \text{ mm}^2$ on days 5, 10, 15, and 20, respectively, which were not significantly different from the myocyte-depleted group at corresponding time points (0.43 ± 0.13 , 0.25 ± 0.03 , 0.21 ± 0.04 , and $0.18 \pm 0.04 \text{ mm}^2$, respectively). Supplementation of myocyte-depleted ECTs with MSCs resulted in a significantly decreased CSA on days 5 and 10 ($p < 0.001$), with

FIG. 3. Double-labeling immunofluorescence of representative sections for three ECT groups at culture day 20: **(A)** baseline ECT composed of 15 M cells/mL, **(B)** myocyte-depleted ECT with half the amount of cells (7.5 M cells/mL), and **(C)** myocyte-depleted ECT (7.5 M cells/mL) supplemented with mesenchymal stem cell (MSC; 0.75 M cells/mL). Grayscale confocal images reveal sarcomeres in α -actinin-expressing cells, and a punctate pattern of connexin 43 (Cx43) expression. In merged images, Cx43 (green) often localizes along the boundaries of α -actinin-positive (red) cells. Nuclei are counterstained with 4',6 diamidino-2-phenylindole (DAPI; blue). Scale bar = 25 μ m. Color images available online at www.liebertpub.com/tea

average values of 0.21 ± 0.02 , 0.12 ± 0.35 , 0.13 ± 0.02 , and 0.16 ± 0.01 mm² on days 5, 10, 15, and 20, respectively.

Developed force per myocyte

Because ECT groups differed in the number of cells capable of developing contractile force, we analyzed the impact of the addition of MSC using a measure of the developed force per myocyte. This analysis was done by dividing the developed force by the total number of myocytes (in millions) used to create the ECT; 1.5 (million) for baseline ECT and 0.75 (million) for myocyte-depleted and myocyte-depleted + MSC groups (Fig. 8). Baseline ECT had average developed forces per million myocytes of 14.1 ± 10.3 , 29.7 ± 22.1 , 29.2 ± 15.9 , and 22.1 ± 13.7 μ N/cell $\times 10^6$ on days 5, 10, 15, and 20, respectively. Although not statistically significant, myocyte-depleted ECT tended to have lower average developed forces of 6.5 ± 3.5 , 9.4 ± 3.1 , 16.9 ± 10.7 , and 41.2 ± 36.2 μ N/cell $\times 10^6$ at matched time points. Compared with the myocyte-depleted group, supplementation with MSCs resulted in significantly increased developed force per million myocytes at days 5 through 15, with corresponding values of 14.9 ± 6.6 μ N/cell $\times 10^6$ ($p = 0.024$), 44.6 ± 30.7 μ N/cell $\times 10^6$

($p = 0.002$), and 33.6 ± 13.7 μ N/cell $\times 10^6$ ($p = 0.039$), while the comparison at day 20 did not quite achieve statistical significance (71.6 ± 17.6 μ N/cell $\times 10^6$, $p = 0.099$).

Pacing threshold

Electrophysiological function of ECT was assessed in terms of VT gradient required for pacing at 2 Hz. On day 5, the myocyte-depleted group had a pacing threshold of 0.25 ± 0.03 V/mm, which was significantly higher than the baseline group (0.19 ± 0.02 V/mm, $p = 0.042$), indicating greater resistance to pacing with fewer cardiomyocytes. The MSC-supplemented group showed a significant reduction in pacing threshold compared with the myocyte-depleted group (0.17 ± 0.02 V/mm, $p = 0.016$), which was not different than baseline ECT. This difference remained significant at day 10 ($p = 0.019$), and the trend continued for the remainder of time in culture (Fig. 9).

Discussion

The therapeutic potential of MSCs has been intensely studied. Over the past decade, several *in vitro*^{15,16} and large

FIG. 4. Cell number and distribution within ECT. **(A)** Hematoxylin and eosin stain of representative sections from each group at day 20 shows good tissue integrity with cells distributed throughout the ECT. Scale bar=50 μ m. **(B)** Confocal microscope images of DAPI-stained nuclei in full-width ECT sections at culture day 20. Scale bar=100 μ m. **(C)** Cell number determined by counting DAPI-stained nuclei in full-width images (as in panel **B**) of one representative ECT per group at each time point. Mean values and standard deviations describe data from three locations (left, middle, and right) along each tissue sample. **(D)** Distribution of cell data presented in panel **C**. Three bars for each tissue group indicate the number of nuclei in three adjacent zones (edge, center, and edge) spanning the width of each representative ECT per time point. Means and standard deviations determined as in panel **C**. Color images available online at www.liebertpub.com/tea

animal studies^{17,18} have supported the use of MSCs as a cell-based therapy for cardiac disease. Additionally, proof-of-concept and phase-I clinical trials have demonstrated the ability of MSCs to improve left ventricular function, induce reverse remodeling, and decrease the scar size after MI in humans.^{19–21} Despite extensive basic and translational research, the specific mechanism of MSC-mediated cardiac repair remains uncertain and highly debated. Candidates include engraftment and differentiation of MSCs into cardiomyocytes,²² MSC stimulation of cardiac stem cell proliferation and differentiation,²³ enhanced angiogenesis,²⁴ and possible MSC-secreted paracrine factors that increase cardiac contractility.²⁵

Elucidating specific repair processes of cell therapies in animal models is difficult for several reasons. Cell retention is an issue, as >90% of injected cells are typically lost.²⁶ Additionally, when evaluating cardiac function after cell therapy, it is difficult to separate direct effects on cardiomyocyte function from indirect processes such as recruitment of endogenous repair mechanisms, angiogenesis, or enhanced survival of resident cells. Because both direct and indirect factors may contribute to the functional benefits of MSC treatment, a simplified *in vitro* model system with reduced and controlled biological complexity may be beneficial in identifying, understanding, and optimizing the therapeutic potential of candidate cell therapies for cardiac disease.

FIG. 5. Quantification of cell orientation over time in ECT. (**A**, **B**) Confocal immunofluorescence images of representative full-width ECT sections from each group labeled with rhodamine-phalloidin at culture days 5 (**A**) and 20 (**B**). Scale bar = 100 μ m. (**C**) Example image processed with the MatFiber script used for measuring cell alignment. Mean vector length, $r=0.75$, in this example. (**D**) Mean vector length for three ECT groups from 48 h to 20 days, indicating increasing cell alignment with time in culture. Mean values and standard deviations describe data from three locations (left, middle, and right) along one representative ECT from each group per time point.

The purpose of this study was to investigate ECT as an *in vitro* model for assessing the effects of MSCs on myocardial contractile function. The myocyte-depleted ECTs, created with 50% fewer NRCM than baseline, demonstrated lower DS throughout the course of the study, constituting a model of impaired cardiac function. The 50% deficit of cardiomyocytes was effectively compensated when myocyte-depleted tissues were supplemented with 10% MSC. The benefit of MSCs on ECT contractile function remained sig-

nificant when data were expressed as developed force per myocyte, suggesting the increased stresses (i.e., force/area) were not simply an artifact of smaller CSA due to MSC-mediated tissue compaction, but reflected genuine improvement of myocyte contractile strength.

To investigate cell type specificity of the observed functional enhancement, a follow-up study specifically compared MSC-supplemented ECTs ($n=6$) with a new group of myocyte-depleted ECTs supplemented with 10% rat dermal

FIG. 6. Developed stress (Pa) versus days in culture for three ECT test groups: (1) ■ baseline, (2) □ myocyte-depleted, and (3) ■ myocyte-depleted+MSC supplement. The myocyte-depleted group showed a significantly decreased developed stress compared to baseline, demonstrating that depletion of cardiomyocytes had a detrimental effect on ECT contractile properties. Supplementing myocyte-depleted ECT with a 10:1 ratio of neonatal rat cardiomyocyte:MSC resulted in restoration of contractile function with significantly enhanced developed stress at days 5 through 15 compared to the myocyte-depleted group. Sample size (*n*) indicated for all time points. Bars indicate standard deviation. ***p* ≤ 0.01 and ****p* ≤ 0.001.

fibroblasts (DF; *n*=16) (Cell Applications, Inc., San Diego, CA), which are not known to have any therapeutic benefit for myocardium. DF compacted ECTs even more strongly than MSCs, becoming so thin that they ruptured before day 15, precluding the analysis over the full-study time course. Nevertheless, some insights may be gained from the early time points (Fig. 10). At culture day 5, average CSAs for the two groups were nearly identical (<3% difference), yet the DF-supplemented group generated <50% of the developed force and stress achieved by MSC-supplemented ECT

FIG. 7. Cross-sectional area (CSA) (mm²) versus days in culture. CSA decreased from day 5 to 20, indicating tissue compaction with time in culture. Supplementation with MSCs resulted in faster compaction and a trend toward smaller CSA compared with baseline and myocyte-depleted groups. Sample size (*n*) as in Figure 6. **p* < 0.05, ***p* ≤ 0.01, and ****p* ≤ 0.001.

FIG. 8. Developed force/myocyte (μN/cell × 10⁶) versus days in culture. The myocyte-depleted + MSC group showed significant enhancement in developed force per million myocytes compared to the myocyte-depleted group at days 5, 10, and 15, and were significantly higher than baseline ECT on day 20. Sample size (*n*) as in Figure 6. **p* < 0.05 and ***p* ≤ 0.01.

(DF:MSC ratio = 0.46 ± 0.15 and 0.47 ± 0.14, *p* = 0.0002 and 0.0004, for force and stress, respectively). At day 10, the DS was less disparate (DF:MSC ratio = 0.65 ± 0.27; *p* = 0.09), as the DF-supplemented group became significantly thinner than MSC-supplemented ECTs (*p* = 0.0002); however, developed force in DF-supplemented ECTs remained <50% of the MSC group (DF:MSC ratio = 0.46 ± 0.16; *p* = 0.0095). Thus, while greater tissue compaction directly impacts calculations of force/area (i.e., stress) and may confer benefits from improved structural alignment or cell-cell contact, the majority of functional enhancement seen with the MSC-supplemented ECTs (Fig. 6) and corroborated by the developed force data (Fig. 8) appears to be an effect specific to the MSCs. This follow-up study also alleviates concerns about possible confounding effects from fibroblast contamination of the MSC population during primary cell isolation.

FIG. 9. Pacing threshold versus days in culture for three ECT test groups. The threshold voltage gradient required for electrical pacing at 2 Hz was significantly lower in the myocyte-depleted supplemented with the MSC group compared to the myocyte-depleted group at days 5 and 10. Sample size (*n*) indicated for all time points. **p* < 0.05.

FIG. 10. Comparison of dermal fibroblast (DF)-supplemented (□) versus MSC-supplemented myocyte-depleted ECT (■). Mean $\pm$ standard deviation values for CSA, DS, and developed force on culture days 5 and 10 were normalized by corresponding values for MSC-supplemented ECT at matched time points. Developed force was significantly lower in DF-supplemented ECT versus MSC-supplemented ECT at both time points, despite greater compaction of ECT by DF at day 10, indicating cell-type specificity of functional enhancement. Sample size (n) as indicated. ** $p \leq 0.01$ and *** $p \leq 0.001$.

In our main study, the benefit of MSC co-culture persisted for 15 days, but enhancements in DS and force per myocyte lost significance at day 20. Similar transient enhancement of the left ventricular ejection fraction (LVEF) in a rat model of MI injected directly with MSCs showed significance at 4 weeks, but not at 6 months,²⁷ implicating an early paracrine effect. Likewise, the BOOST trial, in which patients with MI were treated with bone marrow cell isolates, demonstrated a transient improvement in the LVEF compared to controls, which was statistically significant at 6 months,²⁸ but lost significance at 18- and 60-month follow-ups²⁹ for reasons that remain unclear. The disparities in timing may reflect differences in the number of MSCs used, differences in microenvironment between ECTs and adult cardiac tissue, and species-dependent differences. Identifying and understanding such similarities and differences will be essential for translating results from ECTs to animals and humans.

To examine the structural basis of the observed trends in ECT function, quantitative histology provided data on the cell number, distribution, and alignment over time. The cell number was relatively constant in myocyte-depleted and MSC-supplemented ECTs, suggesting minimal cell death or proliferation from day 5 to 20. In contrast, the cell number decreased over time in baseline ECT (Fig. 4), likely contributing to their attenuated function, which approached the myocyte-depleted group by day 20. A recent study using similar ECT reported substantial cell loss during the week after tissue creation³⁰; our data suggest this may also depend on the initial cell seeding density. Accounting for these trends in the cell number would boost the force/myocyte at later time points for baseline ECT, but the myocyte-depleted and MSC-supplemented ECTs would be minimally affected. There were no obvious trends in cell distribution with the

ECT group or culture time that would explain the patterns in contractile function. Moreover, the distributions did not reveal a preferential cell loss in the central core of the tissue at the early time points, which argues against hypoxia-driven necrosis. Cells became more highly aligned with time in culture for all ECT groups, which likely contributed to the general increase in function over time. The largest increase in cell alignment occurred from day 5 to 10 for the MSC-supplemented ECTs, coincident with the greatest increase in the contractile function.

MSCs and cardiomyocytes can form functional gap junctions in co-culture,³¹ and while some claim these may improve conduction,³² others have suggested detrimental sodium channel inactivation in MSC-coupled cardiomyocytes due to the mismatched resting membrane potential.³³ Our data suggest MSC-supplemented ECTs exhibit enhanced tissue excitability compared to similar ECTs without MSCs, as evidenced by the lowered minimum voltage required for electrical pacing. Further studies using optical mapping or single cell-patch clamping³⁴ will be necessary to clarify the electrophysiological impact of MSC co-culture in ECTs.

This study is not without limitations. The myocyte-depleted ECT condition does not recapitulate the complex process of heart failure; nevertheless, functional characteristics were significantly impaired relative to baseline ECT, thus providing a model of cardiac dysfunction for comparison with our MSC treated group. Secondly, the baseline ECT produced subphysiologic contractile forces, which may limit direct extrapolation to adult myocardium. Similar ECT studies have demonstrated that contractile function can be maximized by optimizing the culture conditions, including the use of unpurified neonatal heart cells.⁶ However, for the present study, it was considered more important to use well-characterized cell populations than to maximize the baseline contractile force. Third, the use of neonatal cardiomyocytes rather than adult cardiomyocytes also limits this study. However, neonatal cardiomyocytes have become the *de facto* standard for cardiac tissue engineering, because adult cardiomyocytes have not been successfully incorporated into 3D matrix cultures. Induced cell maturation in ECTs is an area of ongoing investigation.³⁰ There was a decrease in the sample size with time in culture due to some of the tissues eventually pulling off or tearing away from the posts. Reanalyzing the stress and force data taking into account only those tissues that survived to day 20 did not substantially impact the findings and would not alter the main conclusions of the study. In the future, this problem may be improved by modifying the device to facilitate ECT anchoring onto the posts.^{10,35} Additionally, the inherent variability of primary cells harvested from multiple different donors contributed to the large variability within individual groups of tissues. Miniaturizing the PDMS device to yield more tissues from fewer cells could help reduce this source of variability. ECTs lack a circulatory system, which excludes several potential mechanisms for MSC-based repair that may occur *in vivo*,⁵ but focuses on direct cellular interactions, which appear to be cell type specific. Finally, although collagen type I is a major constituent of the normal myocardium, the natural extracellular matrix is substantially more complex and may impact the applicability of our results.

Conclusion

By utilizing an experimental model with reduced and controlled biological complexity, this study provides direct evidence that MSCs have the intrinsic potential to enhance cardiomyocyte contractility. A 10% supplementation of MSCs compensated for a 50% reduction in the cardiomyocyte number, which was not due to cell proliferation; however, the effect seems to be transient mimicking recent clinical trials. There was also no evidence of detrimental effects on electrical excitability, although extensive electrophysiological characterization is warranted. This study demonstrates that ECT co-cultures can provide a model niche environment that is conducive to evaluating cell therapies for cardiac repair. Future investigation of the mechanisms underlying the observed MSC-associated functional enhancements, including paracrine signaling and cell fusion, will help to translate experimental findings to successful clinical interventions. ECTs provide a unique platform by which to elucidate such mechanisms, filling a critical gap between the Petri dish and the laboratory animal.

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Disclosure Statement

No competing financial interests exist.

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