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Anisotropic microfibrinous scaffolds enhance the organization and function of cardiomyocytes derived from induced pluripotent stem cells

Maureen Wanjare^{1,2}, Luqia Hou^{1,2}, Karina H. Nakayama^{1,2}, Joseph J. Kim^{1,2}, Nicholas P. Mezak¹, Oscar J. Abilez², Evangeline Tatzalos², Joseph C. Wu², and Ngan F. Huang^{1,2,3,*}

¹Center for Tissue Regeneration, Repair and Restoration, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA, USA

²Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA

³Department of Cardiothoracic Surgery, Stanford University, Stanford, CA, USA

Abstract

Engineering of myocardial tissue constructs is a promising approach for treatment of coronary heart disease. To engineer myocardial tissues that better mimic the highly ordered physiological arrangement and function of native cardiomyocytes, we generated electrospun microfibrinous polycaprolactone scaffolds with either randomly oriented (14- μ m fiber diameter) or parallel-aligned (7- μ m fiber diameter) microfiber arrangement and co-seeded the scaffolds with human induced pluripotent stem cell-derived cardiomyocytes (iCMs) and endothelial cells (iECs) for up to 12 days after iCM seeding. Here we demonstrated that aligned microfibrinous scaffolds induced iCM alignment along the direction of the aligned microfibers after 2 days of iCM seeding, as well as promoted greater iCM maturation by increasing the sarcomeric length and gene expression of myosin heavy chain adult isoform (MYH7), in comparison to randomly oriented scaffolds. Furthermore, the benefit of scaffold anisotropy was evident in the significantly higher maximum contraction velocity of iCMs on the aligned scaffolds, compared to randomly oriented scaffolds, at 12 days of culture. Co-seeding of iCMs with iECs led to reduced contractility, compared to when iCMs were seeded alone. These findings demonstrate an dominant role of scaffold anisotropy in engineering cardiovascular tissues that maintain iCM organization and contractile function.

INTRODUCTION

Cardiovascular disease is the leading cause of death in the US. In particular, over 15 million people suffer from coronary heart disease (CHD),¹ which is characterized by narrowing of the coronary arteries that supply blood flow to the heart. CHD leads to the loss of cardiomyocytes, the contractile cells of the heart, and result ultimately in heart failure.²

*Address for Correspondence: Ngan F. Huang, PhD, Assistant Professor, Department of Cardiothoracic Surgery, Stanford University, Address: 300 Pasteur Drive, Stanford, CA 94305-5407, Tel: (650) 849-0559, Fax: (650) 725-3846, ngantina@stanford.edu.

AUTHOR CONTRIBUTIONS

M.W., L.H., K.N. J.J., N.M., and N.H. designed and carried out experiments, and analyzed data. M.W. and N.F.H. interpreted the results. O.A., E.T., and J.C.W. provided technical support of iCM generation and characterization. M.W. and N.H. wrote and organized the manuscript, with editorial input from L.H., K.N., and J.C.W.

Current cell-based clinical trials to restore the function of the damaged cardiomyocytes by local delivery of therapeutic cells have shown only moderate benefit in improving cardiac pumping capacity.³ A contributing factor to the limited therapeutic effect of implanted cells is ineffective electromechanical coupling. Primary cardiomyocytes have highly organized physiological structure with ordered cellular alignments, and this structure is critical to driving efficient contractility.

Tissue engineering is a promising approach to mimic the well-ordered cellular composition and architecture of the native myocardium. Parallel-aligned (anisotropic) polymer scaffolds can provide contact guidance to cells to reorganize cellular orientation and function. Current approaches to fabricate aligned scaffolds include mechanical loading,⁴ microfluidic alignment,^{5, 6} flow and magnetic field induced alignment,⁷ electrochemical processing,⁸ interstitial flow,⁹ high magnetic field,^{10, 11} oriented electrospinning,¹² melt electrospinning,¹³ Langmuir-Blodgett deposition,¹⁴ extrusion,^{15, 16} and spin-coating.¹⁷ The aligned scaffolds produced by these methods provided for essential interactions between cells and extracellular matrix (ECM) that successfully guided cardiomyocyte alignment.¹⁸

In particular, electrospinning enables the fabrication of microfibrinous scaffolds over a large area with controlled fiber structure and fiber diameter, which is important for scalability and clinical translation. Electrospinning can be used to generate scaffolds derived from natural or synthetic polymers. Natural polymers such as collagen and gelatin possess weak mechanical properties, rapid degradation rates, and can be denatured when dissolved in organic solvents.¹⁹ On the other hand, synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) and poly(caprolactone) (PCL) provide tunable mechanical properties and stability in the body, although they lack biological cell binding sites.¹⁹ PCL has been widely studied and clinically established as biodegradable and biocompatible.²⁰ Additionally, PCL is favorable for cardiac tissue engineering owing to the polymer's flexible mechanical properties, non-toxic nature, and cytocompatibility.²⁰ Since a high surface area to volume ratio and interconnected pores are often desired to ensure optimal oxygen and nutrient transport, polyethylene oxide (PEO) nanofibers are often electrospun concurrently with PCL polymers as a sacrificial component,²¹ resulting in a greater porosity for cellular infiltration upon removal of the PEO using aqueous solvents.²¹

Besides the structural organization of the myocardium, the cellular composition is also an important modulator of cardiomyocyte function. In the native myocardium, adjacent to every cardiomyocyte is a blood vessel that provides nutrients and oxygen to the cardiomyocytes.²² During development, the vascular endothelial cells that comprise blood vessels are known to influence myocardial cell maturation and function.²² Accordingly, cell-cell interactions between cardiomyocytes and endothelial cells may be important to simulate the cellular composition of the native myocardium in engineered cardiovascular tissues.^{23, 24}

A feasible cell source for the generation of cardiomyocytes and endothelial cells are human induced pluripotent stem cells (iPSCs). The iPSCs have nearly unlimited self-renewal capacity and can differentiate into cells of all three germ layers, including cardiovascular lineages.^{25–29} Consequently, iPSCs can theoretically give rise to an infinite number of

autologous iPSC-derived cardiomyocytes (iCMs) and iPSC-derived endothelial cells (iECs) for tissue engineering and cell therapy applications.

Accordingly, the goal of this study was to engineer myocardial tissue constructs that better mimic the native structure and contractile function of cardiomyocytes. In this study, iCMs were cultured on either randomly oriented or parallel-aligned microfibrinous scaffolds to evaluate the effect of scaffold anisotropy on iCM organization and contractile function. To further reveal the role of cell-cell interactions, iECs were co-seeded with iCMs to evaluate the combined effect of cell-scaffold and cell-cell interactions on iCM function.

EXPERIMENTAL

Polymer Preparation and Electrospinning

Microfibrinous PCL scaffolds with either randomly oriented or parallel-aligned fiber orientation were fabricated by electrospinning in the presence of PEO sacrificial polymer (Suppl. Fig. 1). PCL (30% weight/volume PCL, Sigma Aldrich, M_n 80,000) and PEO (10% weight/volume, Sigma Aldrich, M_v 100,000) were simultaneously electrospun from separate syringes at 3.5 ml/h and 1.0 ml/h, respectively, using a commercial electrospinner (Nanospinner 24-XP, Inovenso Ltd.) at a voltage of 25.0 kV, mandrel speed of 200 RPM, and a distance from spinneret to mandrel of 100 mm. After electrospinning, the randomly oriented microfibrinous scaffold sheet was desiccated for 24 hours at room temperature. The aligned microfiber orientation was generated from the randomly oriented scaffold using heat-based mechanical rearrangement of fiber organization.³⁰ Briefly, segments of the scaffold sheet ($12 \times 25 \times 0.1$ mm) were stretched uniaxially to 300% of initial length using a 1 kg weight for 1 hour at 55°C. The scaffolds treated with uniaxial stretching were subsequently denoted as the aligned scaffolds. After fabrication of both randomly oriented and aligned fiber orientation scaffolds, circular discs were cut from the scaffold sheets using a 6 mm biopsy punch (Medex Supply). The resulting scaffold discs (6 mm diameter, 0.1 mm in thickness) were submerged in double distilled water (ddH₂O) at 37°C overnight with agitation to dissolve and remove the PEO from PCL. The following day, scaffold discs were transferred to 70% ethanol and incubated with agitation at room temperature overnight for sterilization for 18 hours. After ethanol sterilization, scaffold discs were washed three times in sterile phosphate buffered saline (PBS, Invitrogen) before pretreated in Geltrex (Sigma, 1:200 dilution) ECM proteins with agitation for 18 hours to facilitate cell attachment onto the scaffolds.

Scaffold Characterization

After removal of the sacrificial PEO, the acellular PCL scaffolds with randomly oriented or aligned microfibrinous structure were characterized for structural and mechanical properties. To compare the pore size and porosity between the randomly oriented and the aligned fibrous scaffolds, the scaffolds were imaged by confocal microscopy using an emission wavelength of 460–490 nm, owing to the intrinsic fluorescence of the microfibers in this emission window. Using the confocal microscopy images obtained from randomly oriented and the aligned fibrous scaffolds, the area (A) of each void was quantified by ImageJ

software, and the equivalent diameter (D) was calculated as $2X \sqrt{\frac{A}{\pi}}$ (n=3).³¹ For measurement of porosity, the void area within each image was analyzed by ImageJ and expressed as a percentage of total area (n=3). The angle of orientation was quantified from confocal microscopy images using FibriTool, an ImageJ macro (n=3).³² The angle of orientation ranged from 0° (parallel) to 90° (orthogonal), with respect to the horizontal axis. From the images, fiber diameters were measured using the line tool (ImageJ) by drawing a line across the fibers to generate an intensity plot. The fiber diameter was calculated as the length spanning the histogram, as measured from the base (n=3).

Mechanical uniaxial tensile testing was conducted using an Instron uniaxial testing instrument (Instron 8511, Canton, MA) to determine the tensile elastic modulus, elongation at break and maximum tensile strength. Dog-bone shaped scaffolds (1.5 cm × 1cm) with either randomly oriented or aligned microfibers were subjected to a strain rate of 5 mm/minute using a 5N load cell until break (n=3). For anisotropic samples, the axis of strain was parallel to the direction of the microfibers.

Human iPSC Culture

The human iPSC line (strain P356 provided by Dr. Joshua Knowles, MD, PhD), was generated by reprogramming of healthy human peripheral blood mononuclear cells using Sendai virus-mediated transduction of Sox2, Oct3/4, KLF4, and c-myc.³³ The iPSCs were passaged every four days using ethylenediaminetetraacetic acid (0.1 mM, EDTA) dissociation reagent (Invitrogen). The cells were expanded on tissue culture dishes coated with Geltrex basement membrane matrix proteins (1:200 dilution, Sigma) in Essential 8 expansion medium (Invitrogen). All cells were cultured in humidified incubators at 37°C and 5% CO₂.

Generation of iCMs

Human iPSCs were differentiated into iCMs using an established differentiation procedure.³⁴ In brief, iCMs were generated using a directed differentiation method that involved incubating iPSCs with Wnt agonist CHIR 99021 (6 μM, Selleck), followed by Wnt antagonist IWR-1 (5 μM, Selleck) in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher) supplemented with insulin-free B27 (Invitrogen). After 7 days, the culture medium was replaced with RPMI 1640 supplemented with B27 and insulin (Invitrogen). To enrich for iCM populations, the differentiating cells were grown in RPMI 1640 lacking D-glucose (Invitrogen) after 9 days. The iCMs were used for experiments after 15 days of differentiation. Dishes containing >80% spontaneously contracting iCMs were used for the described experiments.

Generation of iECs

Induction of endothelial differentiation was carried out using an established protocol.³⁵ Briefly, the iPSCs were supplemented with Wnt agonist CHIR 99021 (5 μM, Selleck), bone morphogenetic protein-4 (BMP4, 25 ng/mL, Peprotech), B27 supplement (Gibco), and N2 supplement (Gibco). After 3 days, the cells were dissociated with HyQase (Fisher

Scientific) and plated at a density of 3.3×10^4 cells/cm² in StemPro media (Gibco), supplemented with forskolin (5 μ M, LC Labs), vascular endothelial growth factor (VEGF, 50ng/mL, Peprotech), and polyvinyl alcohol (2 mg/mL, Sigma). After 7 days, the cells were washed twice with PBS, and cultured in endothelial growth media (EGM-2MV, Lonza) supplemented with additional VEGF (100 ng/ml) for 7 more days.

Flow Cytometry Analysis of iECs

After differentiation, iECs were dissociated with HyQtase and blocked with 0.1% bovine serum albumin (BSA, Invitrogen). Cells were incubated with fluorescein (FITC)-conjugated anti-human CD31 antibody (eBioscience) for 15 min at 4°C. An isotype-matched antibody was used as a negative control. After incubation, the cells were washed with 0.1% BSA, incubated with propidium iodide (1 μ g/ml, Invitrogen) and then analyzed using a FACS Aria (BD) flow cytometer. Further quantitative analysis was performed using FlowJo software (Tree Star).

Cell Seeding Onto Scaffolds

Prior to cell seeding, the randomly oriented and aligned scaffold discs (6 mm in diameter, 0.1mm in thickness) were sterilized in 70% ethanol, washed in PBS, and incubated Geltrex basement membrane proteins (1:200 dilution) for 12 hours at 37°C. For scaffolds cultured with iCMs only, the iCMs were dissociated from tissue culture dishes after differentiation using 0.05% trypsin-EDTA (Gibco), and then seeded onto the randomly oriented or aligned scaffold groups at a density of 1×10^6 cells per scaffold in iCM culture medium consisting of RPMI 1640 containing B27 and insulin. For scaffolds seeded with both iCMs and iECs, the cells were seeded in a sequential fashion (Fig. 1A). First, iECs were dissociated from cell culture dishes using HyQtase and replated onto the scaffolds at a density of 4×10^4 per scaffold in EGM-2MV endothelial expansion media on day -3. After 3 days (day 0), iCMs were then seeded onto either randomly oriented or aligned scaffolds at a density of 10^6 cells per scaffold in iCM culture medium. The cell-seeded scaffolds were harvested after a total of 2 days or 12 days from the time of iCM seeding for analysis of iCM function. The seeding densities of iCMs and iECs were based on previous studies that suggest >8-fold more cardiomyocytes than endothelial cells in the native myocardium of mice.³⁶

Immunofluorescence Staining

Immunofluorescence staining was performed to visualize the iCMs and iECs within the scaffolds using phenotypic markers. The contractile protein troponin-T was used to distinguish iCMs from other cells. The endothelial cell adhesion molecule, CD31, was used to distinguish iECs from other cell types. Furthermore, gap junctions were assessed by connexin-43. The cell-seeded scaffolds were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), permeabilized in 0.1% Triton X-100 (Sigma-Aldrich), and then blocked with 1% BSA.^{37, 38} The scaffolds were then incubated with primary antibodies directed against anti-human troponin-T (Abcam), anti-human connexin-43 (Sigma), and anti-human CD31 I (Dako), followed by secondary antibodies conjugated to AlexaFluor 488 or AlexaFluor 594 (both from Thermo Fisher Scientific). The samples were washed in PBS, and total nuclei were counterstained using Hoechst 33342 (Thermo Fisher Scientific) nuclear dye. Samples were stored at 4°C in the dark and imaged using a laser scanning

confocal microscope (LSM710, Zeiss). Based on troponin-T immunofluorescence staining, the angle of alignment of iCMs was quantified manually by ImageJ with respect to the axis of the microfiber orientation, in which an angle of 0° approximates perfect alignment relative to the fibers (n=3).^{30, 38} Sarcomeric length, which indirectly is a measure of active force generation in iCMs, was quantified using the line tool in Image J and plotted as a histogram of fluorescence intensity. The sarcomeric length was calculated as the distance between adjacent troughs, which corresponds to the distance between Z-lines within the myofibrils of iCMs (n = 4).³⁹

iCM Contractility Measurements

The effect of scaffold anisotropy on iCM function and health was quantified based on their contractility under spontaneous or electrically stimulated (1 Hz) conditions at 2 and 12 days after iCM seeding (n = 3). The contractile properties of iCMs were assessed under a fluorescence microscope (Nikon Eclipse Ti-E) using a video-based cell geometry system. To better visualize the cells within the scaffolds, the cells were pretreated with calcein-AM (2 µm) fluorescent vital dye for 15 minutes prior to acquisition of contractility videos. For electrically stimulated conditions, the cell-seeded scaffolds were positioned between a pair of carbon electrodes that was submerged into RPMI 1640 media. The scaffolds were field stimulated to contract by the MyoPacer field stimulator (IonOptix) at a frequency of 1.0 Hz, pulse duration of 4 ms, and amplitude of 30 V. Three randomly selected regions of interest were selected from each scaffold for video recording. Each video was acquired under 10x objectives at 16 frames per second for at least 15 s. For contraction analysis, the movies in the form of .tiff series were entered into a custom Matlab code developed by Huebsch *et al.*⁴⁰ that generated motion vector fields. A macroblock size of 16 pixels and a maximum detectable motion of 7 pixels was selected. The code generated one-dimensional tracings of contraction speed, which were used to generate contraction heatmaps and beating speed histograms, along with the computed values of . the maximum contraction velocity (µm/s) and periodicity of contraction (expressed as beats/minute).

Quantitative Polymerase Chain Reaction

Gene expression analysis was conducted using RT-PCR of cDNA synthesized from purified RNA. The GeneJET RNA purification kit (Thermo-Fisher Scientific) was used to isolate and purify total RNA, according to the manufacturer's instructions. The concentration of total RNA was measured using a UV-Vis Spectrophotometer (NanoDrop 2000, Thermo Scientific), and cDNA was synthesized from total RNA using the SuperScript II First-Strand cDNA Synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions and synthesized in a compact thermal cycler (T100 Thermal Cycler, Bio-Rad). Real-time PCR was carried out using Taqman primers (Thermo Fisher Scientific) for troponin-T2 (TNNT2), α-myosin heavy chain (MYH6), β-myosin heavy chain (MYH7), calsequestrin (CASQ2), and GAPDH using a 7300 Real-Time PCR System (Applied Biosystems) with the following thermal profile: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds/cycle, and then 60°C for 1 minute. The data were quantified by the Ct method,⁴¹ normalized to GAPDH housekeeping gene, and then expressed as relative fold changes (n = 3).

Statistical Analysis

Data are shown as mean $\pm$ standard deviation. A Student's t-test was used for comparisons between two groups. A Kruskal-Wallis analysis of variance (ANOVA) with Holm's adjustment was performed for assessment of sarcomeric length between treatment groups. For the other remaining studies, a one-way ANOVA tests were performed, with Holm's adjustment for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of Mechanical Properties and Anisotropy of Microfibrous Scaffolds

The randomly oriented microfibrous scaffolds were prepared by electrospinning (Fig. 1B). By further applying uniaxial stretch to the scaffolds, the microfibers reorganized into parallel-aligned anisotropic scaffolds (Fig. 1C, Suppl. Fig. 1). The average fiber diameter of a randomly oriented scaffolds was $14.3 \pm 1.3 \mu\text{m}$, whereas the corresponding diameter for the aligned scaffolds was $7.0 \pm 0.5 \mu\text{m}$. The equivalent pore diameter ($\sim 19\text{--}22 \mu\text{m}$) was similar between the two scaffolds (Table 1). Likewise, the porosity of the randomly oriented scaffold was $34.7 \pm 11.2\%$, which was similar to that of the aligned scaffold. However, owing to the uniaxial pre-stretching regimen to generate the aligned scaffolds, the aligned scaffolds had a lower elongation at break of $82.2 \pm 6.4\%$, in comparison to $426.3 \pm 44.0\%$ for the randomly oriented scaffolds (Table 1). In addition, the aligned scaffolds had a two-fold increase in Young's Modulus and three-fold increase in the maximum tensile strength, compared to the randomly oriented scaffold, presumably due to the anisotropic organization of the microfibers.

To quantify the degree of anisotropy in the scaffolds, the angle of orientation between the randomly oriented and aligned scaffolds was measured. The degree of alignment computes the mean orientation of the microfibers, relative to the horizontal axis. The degree of alignment of the randomly oriented scaffold was $50 \pm 29^\circ$ with respect to the horizontal axis, which suggested a range in the distribution of microfiber orientations. In contrast, the angle of orientation of the aligned scaffold was $5 \pm 4^\circ$, which was significantly lower ($P < 0.05$) and suggested that the fibers in the aligned scaffolds were highly organized in close proximity to the axis of the horizontal axis, which coincided with the axis of the aligned microfibers (Fig. 1D). These quantitative results validated the observed anisotropy of the aligned microfibrous scaffolds.

iCM Cellular Morphology and Organization in Microfibrous Scaffolds

Having characterized the mechanical properties of the microfibrous scaffolds, we next generated iCMs and iECs with $>80\%$ purity, based on immunofluorescence staining (Fig. 1E–F) of phenotypic markers when cultured on tissue culture dishes. For iECs, FACS analysis for further validated the $>80\%$ yield of CD31^+ iECs after differentiation (Suppl. Fig. 2). Without purification, the iCMs and iECs were seeded onto the scaffolds to assess for cellular morphology and organization after 2 days of iCM seeding. Based on immunofluorescence staining of cardiac troponin-T, a contractile protein expressed in iCMs, we could visualize the morphology of iCMs when cultured on the microfibrous scaffold

(Fig. 2A). On the aligned scaffolds, the iCMs were generally elongated and aligned along the direction of the microfibers, although the iCMs tended to organize in larger aggregates of aligned cells. In contrast, the iCMs cultured on the randomly oriented scaffolds lacked order in their cellular arrangement, and tended to aggregate into larger clusters. Similar observations were found on the scaffolds that were cocultured with iECs, in which the iCMs on the aligned scaffolds were highly aligned along the direction of the microfibers. To quantify these observed differences in cellular organization, we measured the degree of iCM alignment, as expressed as the orientation of the cell with respect to the orientation of the microfibers, in which 0–20° indicated an orientation within close proximity to the axis of the microfibers. On the randomly oriented scaffolds seeded with iCMs, the distribution of cellular alignments ranged from 0–90° with a mean degree of alignment of $46.8 \pm 4.3^\circ$, suggesting an unorganized distribution of iCMs (Fig. 2B–C). The coculture of iCMs + iECs on the randomly oriented scaffold led to a similar disorganized cellular arrangement with a corresponding mean of cell alignment of $43.6 \pm 14.3^\circ$ (Fig. 2B–C). In contrast, for the iCMs on the aligned scaffold, the mean degree of alignment of $9.2 \pm 2.5^\circ$, which suggested highly ordered cellular arrangement. In the presence of both iCMs and iECs, the mean degree of alignment on the aligned scaffold was $11.4 \pm 3.6^\circ$, which was similar to the mean degree of alignment on aligned scaffolds in the presence of iCMs alone (Fig. 2B–C). Taken together, these data demonstrated that the iCMs were highly aligned along the direction of the aligned microfibers.

To investigate the electrical coupling between adjacent iCMs, connexin-43, which is a gap junctional protein that mediates intercellular electrical coupling, was stained (Suppl. Fig. 3). We observed a similar degree of punctate connexin-43 expression in iCMs for all four treatment groups, which suggested that iCMs were electrically capable of communicating with each other, regardless of scaffold topography.

iCM Maturation on Aligned Microfibrous Scaffolds

Unlike primary cardiomyocytes that are organized in a highly anisotropic manner *in vivo*, iCMs cultured in cell culture dishes do not maintain an ordered cellular morphology. Furthermore, iCMs are known to have an immature phenotype that resembles a fetal-like state rather than an adult state.^{42, 43} Therefore, we next examined whether the anisotropy of the scaffolds could influence not only iCM cell morphology but also maturity. The sarcomere, the basic contractile unit of a cardiomyocyte, is 1.6–2.2 μm in length in the relaxed state in mature cardiomyocytes.⁴⁴ Immunofluorescence staining of troponin-T was used to quantify the sarcomeric length of iCMs cultured on randomly oriented or aligned microfibrous scaffolds after 2 days of iCM seeding. As shown in Fig. 3A, the sarcomeric length of iCMs on the aligned scaffold ($1.6 \pm 0.1 \mu\text{m}$) was significantly higher than that on the randomly oriented scaffold ($1.3 \pm 0.1 \mu\text{m}$), suggesting that the iCMs on aligned scaffolds resembled cells of greater maturity than those on randomly oriented scaffolds. The presence of iECs on the scaffolds, however, did not appear to have a significant effect on the sarcomeric length, in comparison to scaffolds seeded with iCMs alone.

To verify these results, we performed gene expression analysis against myosin heavy chain (MHC), a structural protein in cardiomyocytes that is known to switch in isoform from

MYH6 (α -MHC) during embryonic development to MYH7 (β -MHC) in adults.⁴⁵ As shown in Fig. 3B–C, the gene expression of MYH7 after 2 days of iCM culture showed significant upregulation on the aligned scaffold ($P < 0.05$), in comparison to the randomly oriented scaffold, and this relationship was maintained after 12 days. Furthermore, another gene associated with mature calcium handling phenotype, CASQ2,⁴⁶ also showed a 4-fold upregulation on the aligned scaffold ($P < 0.05$), in comparison to the randomly oriented scaffold after 12 days, although there were no significant differences at the earlier 2 day time point. The addition of iECs resulted in a significant decrease in the expression of MYH7 and CASQ2 isoform at day 12, when comparing the aligned scaffold seeded with iCMs vs the aligned scaffold seeded with both iCMs and iECs (Fig. 3C). The expression of the MHC fetal isoform, MYH6, along with the contractile marker, troponin-T2 (TNNT2), did not show significant differences between treatment groups at the early time point of 2 days, but after 12 days showed an upregulation on the aligned scaffold ($P < 0.05$), in comparison to the randomly oriented scaffold. Interestingly, among the treatment groups seeded with both iCMs and iECs, the expression of the MYH6 fetal isoform was significantly higher in the randomly oriented scaffold group after 12 days (Fig. 3C). Together, these results suggested that scaffold anisotropy played a dominant role over coculture- with iECs in improving the maturity of iCMs.

iCM Contractility on Aligned Microfibrous Scaffolds

Since contractility is a defining characteristic of cardiomyocytes, we also compared the contractility of iCMs cultured on aligned vs. randomly oriented scaffolds. Using a fluorescent calcein-AM dye to label viable cells on the scaffold, we then acquired fluorescent videos to capture the contractility of iCMs in the spontaneous state or in response to electrical stimulation. Using a video-based analysis of iCM contractility, heat maps depicting the absolute motion were generated automatically using MATLAB code (Fig. 4A–D) to provide an overview of the relative motion that was observed. Representative histograms of the beating speed provide an assessment of the magnitude and periodicity of contractions versus time. Based on spontaneous contractility after 2 days of cell seeding, the histograms depicted the periodicity of contraction to be 27 ± 8 beats/min and 9 ± 1 beats/min for iCMs cultured on either randomly oriented scaffolds and aligned scaffolds, respectively. Spontaneous contraction was not observed with iCMs cultured with iECs, regardless of the scaffold topography. To quantify the magnitude of contractility, the maximum contraction velocity was calculated (Fig. 4E). The maximum contraction velocity was significantly higher on the scaffolds cultured in the absence of iECs, although there was not a significant difference between iCMs cultured on the randomly oriented scaffold (4.0 ± 1.4 $\mu\text{m/s}$) vs aligned (3.6 ± 1.3 $\mu\text{m/s}$) scaffold.

In comparison to cells that were spontaneously contracting on the scaffold, cells that were electrically paced (1 Hz) contracted more regularly and similarly at 60 ± 1 beats/min for iCMs cultured on both randomly oriented and aligned scaffolds in the absence of iECs (Fig. 5A–D). The iCMs cocultured with iECs did not contract upon electrical stimulation, regardless of the scaffold topography. The maximum contraction velocity remained significantly higher in scaffolds seeded with iCMs only (3.8 ± 1.8 $\mu\text{m/s}$ randomly oriented and 2.4 ± 0.9 $\mu\text{m/s}$ aligned), in comparison to those co-seeded with iECs (0.2 ± 0.1 $\mu\text{m/s}$

randomly oriented, and $0.5 \pm 0.2 \mu\text{m/s}$ aligned), (Fig. 5E). However, there was no significant difference in maximum contraction velocity between iCMs cultured on the randomly oriented scaffolds vs aligned scaffolds.

Since iCMs are known to progressively mature in phenotype and function with respect to time in culture,²¹ we further compared the contractility of iCMs after 12 days of culture on the scaffolds. When paced at 1 Hz, the iCMs on the aligned scaffolds showed a fast and more uniform periodicity of contraction (60 ± 1 beats/min) that was similar to iCMs on the randomly oriented scaffold (55 ± 7 beats/min) (Fig. 6A–B). The iCMs on the aligned scaffolds co-seeded with iECs also showed uniform contraction (60 ± 1 beats/min), whereas iCMs on the randomly oriented scaffold co-seeded with iECs failed to contract upon electrical stimulation (Fig. 6C–D). The maximum contraction velocity was significantly higher on the aligned scaffold seeded with iCMs ($5.0 \pm 0.6 \mu\text{m/s}$, in comparison to all other groups (Fig. 6E). Interestingly, the iCMs cocultured with iECs on the aligned scaffold (Fig. 6D) was able to exert measurable contraction velocity ($2.0 \pm 0.8 \mu\text{m/s}$).

In comparison to day 2, the iCMs on day 12 in all treatment groups formed more and larger clusters, leading to areas of the scaffold with low cellularity. The iCMs cultured alone on the randomly oriented scaffolds tended to exert more cell-cell interaction than with the scaffolds, resulting in local areas of aggregation (Fig. 6A). On the aligned scaffolds seeded with iCMs alone or in coculture with iECs, the iCMs on day 12 organized into larger aggregates that generally remained oriented along the direction of the fibers

Taken together, these data suggested that the contractility of iCMs was similar between randomly oriented and aligned scaffolds at early time points of 2 days, but the aligned scaffolds enhanced contraction velocity at a later time point of 12 days after cell seeding.

DISCUSSION

The mechanical and electrical properties of the myocardium largely depend on the spatial organization of myofibers.^{47, 48} Since PCL is biocompatible, biodegradable, and widely studied for tissue engineering applications,²⁰ this polymer was selected as a suitable scaffold to support iCMs.²⁰ The mechanical properties of the PCL scaffolds were within an order of magnitude to the native myocardium. In comparison to the human myocardium that ranges in Young's modulus from 0.2–0.5 MPa,^{49, 50} the randomly-oriented scaffolds had a Young's modulus of 0.72 MPa (Table 1). The anisotropic PCL scaffolds had a Young's modulus of 1.81 MPa that was stiffer than the randomly oriented scaffolds. A potential reason for the increased stiffness was the reduced degree of translation or slip of microfibers over one another since the scaffolds were previously stretched in order to achieve fiber alignment. In addition, both randomly-oriented and aligned scaffolds had similar pore size and porosity. Accordingly, the mechanical properties of the PCL scaffolds appeared suitable for cardiovascular tissue engineering applications.

The organization and function of cardiomyocytes in the myocardium is highly dependent on the topography of the myofibers in the myocardium. Several studies have investigated the influence of fiber topography of scaffolds on cardiomyocyte organization. For example,

Fleischer *et al.* reported that electrospun PCL microfibers (~1.3 –2.8 μm diameter) promoted enhanced spreading and elongation of primary rat cardiomyocytes, when compared to nanoscale (~300 nm diameter) fibrous scaffolds.⁵⁰ Similarly, the alignment of scaffolds seeded with cardiomyocytes alone or co-seeded with fibroblast supporting cells were significantly more aligned than those on randomly oriented scaffolds. Parrag *et al.* reported that embryonic stem cell-derived cardiomyocytes showed significantly improved cell alignment and sarcomere organization on aligned electrospun polyurethane scaffolds, in comparison to randomly oriented scaffolds.⁵¹ These studies corroborate our findings that scaffold anisotropy modulates iCM organization and phenotype.

Contact guidance cues provided by different topographies influence the mechanosignaling of cardiomyocytes. In our study, the aligned scaffolds seeded with iCMs exhibited increased sarcomeric length as well as increased MYH7 gene expression, when compared to iCMs on randomly oriented scaffolds (Fig. 3). As Ribeiro *et al.* have reported, the imposed iCM alignment due to scaffold anisotropy changed iCM cell shape to better resemble the elongated rod-shape of primary adult cardiomyocytes, and subsequently tuned the distribution of intracellular tension needed for sarcomere activity.³⁹ Additionally, the iCM intracellular tension was also altered by the increased contractile MYH7 gene expression, which is an indication of increased cardiomyocyte maturity. Based on our findings, the aligned scaffolds could modulate iCM alignment as well as sarcomere organization. However, the gene expression data is limited by normalization with a general housekeeping gene (GAPDH), which may skew the data from samples in which iECs were present. Accordingly, assessment of the sarcomere ultrastructure should be performed to further validate these results.

After 2 days of culture, iCM organization and function were evident from spontaneous and electrically paced contractions. The scaffolds seeded with iCMs alone had a higher magnitude of beating speed and maximum contraction velocity, compared to scaffolds co-seeded with iCMs and iECs (Fig. 4). When electrically stimulated, the contractions became more synchronous, but the scaffolds seeded with iCMs alone maintained a higher magnitude of beating speed and maximum contraction velocity, compared to scaffolds co-seeded with iCMs and iECs (Fig. 5). However, after 12 days in culture, the iCMs on the randomly oriented scaffolds tended to interact more with each other than the microfibers, forming larger cellular aggregates that could potentially reduce contact guidance cues from the fibers. The reduction in topographical signaling cues could be a reason for the significantly reduced maximum contraction velocity in iCMs cultured on the randomly oriented scaffolds, when compared to those on the aligned scaffolds (Fig. 6). Furthermore, since iCMs are known to progressively mature over time in culture,²¹ iCMs on the aligned scaffolds appeared to resemble a more mature phenotype (Fig. 3C), compared to iCMs on randomly oriented scaffolds, leading to greater contraction velocity after 12 days.

Since endothelial cells are known to provide important cell-cell signaling cues to cardiomyocytes that mediate cell survival and function,²³ we assessed the effect of both scaffold anisotropy as well as co-seeding with iECs on iCM morphology and function. Although iECs supported iCM alignment and morphology on the aligned scaffolds after 2 days of culture, the iECs appeared to reduce contractility of the iCMs, compared to scaffolds

seeded with iCMs alone. It is possible that the iECs disrupted iCM intercellular interactions that led to reduced contraction at early time points of 2 days after seeding. However, after 12 days of cell seeding, the aligned scaffolds seeded with iCMs and iECs contracted periodically with electrical stimulation (Fig. 6D). In contrast, on the randomly oriented scaffolds, the iCMs cocultured with iECs tended to form cellular aggregates and failed to contract with electrical stimulation (Fig. 6C). This finding is consistent with aligned topography providing maturation cues to iCMs that promote cell contractility.

Although other papers have reported that the coculture of cardiomyocytes with endothelial cells could improve cardiomyocyte function and phenotype,^{23, 52} our finding in the reduction of contractility when cocultured with iCMs and iECs may be due to the intercellular composition and ratio, leading to differences in observed contractility. For example, Gao *et al.* showed that multi-cellular culture of iCMs with iECs and iPSC-derived smooth muscle cells at a 2:1:1 relative ratio in a methacrylated gelatin patch could sustain synchronized iCM contractility.⁵³ Furthermore, we and others have previously reported that stromal cells, in addition to endothelial cells, may be required for maintenance of iCM phenotype and function.^{54, 55} The stiffness of the PCL scaffold used in this study could have adversely affected iCM contractility, as compliant hydrogels composed of fibrin have previously shown to support contractility of native cardiomyocytes or those derived from pluripotent stem cells.⁵⁶ The interplay between the mechanical properties and the cellular composition of a scaffold is an issue deserving further investigation. Our results underscore the complexity of cell-cell and cell-scaffold signaling cues that mediate iCM function.

CONCLUSION

In summary, the main findings of this work are that aligned microfibrillar scaffolds 1) induce highly organized iCMs along the direction of the microfibers after 2 days, 2) promote a more mature phenotype based on sarcomere length and gene expression of adult contractile markers, when compared to randomly oriented scaffolds for up to 12 days, and 3) support higher iCM contraction velocity after 12 days. Co-seeding of iCMs with iECs provided limited benefit in supporting iCM contractility after 12 days. These studies highlight the importance of scaffold anisotropy in engineering of cardiovascular tissues for treatment of CHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Overview of experiments and characterization of scaffolds

A. Schematic of experimental design in which iECs and/or iCMs were cultured onto randomly oriented (random scaffold) or aligned microfibrous scaffolds for analysis of morphology, contractile function, and gene expression. B-C. Confocal microscopy images depicting microfibers in randomly oriented (B) or aligned (C) microfibrous scaffolds. D. The angle of alignment of microfibers between randomly oriented or aligned scaffolds (n = 3). E-F. Confocal microscopy images of phenotypic markers troponin-T (red) for iCMs (E) and CD31 (green) for iECs (F) on tissue culture dishes after differentiation to visualize the

efficiency of differentiation. Scale bar = 100 μm . * denotes statistically significant relationship ($P < 0.05$).

Fig. 2. The effect of microfibrillar scaffold anisotropy on iCM alignment after 2 days of iCM seeding

A. Confocal microscopy images of randomly oriented or aligned scaffolds seeded with iCMs alone or iCM + iECs after 2 days of iCM seeding. The iCMs were visualized by troponin-T (TNNT, red), whereas iECs were visualized by CD31 (green). B. Quantification of the angle of iCM alignment (n = 3). C. The distribution of iCM angle of alignment. Scale bar = 100 μ m. Arrow denotes orientation of aligned microfibers. * denotes a statistically significant relationship (P < 0.05).

Fig. 3. The effect of microfibrinous scaffold anisotropy on iCM maturation

A. Quantification of iCM sarcomeric length after 2 days of iCM seeding on randomly oriented (random) or aligned scaffolds, based on immunofluorescence staining of troponin-T (TNNT). Inset shows iCM sarcomeres as stained by troponin-T. Sarcomeres were visualized as parallel lines along the length of the iCM, in which the sarcomeric length was measured as the average distance between adjacent striations (n = 4). Arrows denote two adjacent striations, and the distance between striations is the sarcomere. B-C. Gene expression analysis of iCM contractile markers TNNT2, MYH6, MYH7, and CASQ2 after 2 days (B) or after 12 days (C) (n=3). * denotes significantly different from randomly oriented scaffold

seeded with iCMs. # denotes significantly different from aligned scaffold seeded with iCMs.
\$ denotes a statistically significant relationship ($P < 0.05$).

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Fig. 4. Comparison of iCM spontaneous contractility on randomly oriented or aligned microfibrous scaffolds after 2 days of cell seeding

Cells were fluorescently labeled using calcein-AM (calcein) and corresponding videos of spontaneous contractions (top left) were then acquired. Contractility heat maps (top right) were generated using MATLAB code. A representative histogram of beating speed to measure the periodicity of contraction is depicted (bottom). Comparisons are shown between (A) randomly oriented scaffold + iCM, (B) aligned scaffold + iCM, (C) randomly oriented scaffold + iCM + iEC, and (D) aligned scaffold + iCM + iEC. E. Quantification of maximum contraction velocity among groups ($n = 3$). Arrow denotes orientation of aligned microfibers. * denotes a statistically significant relationship ($P < 0.05$).

Fig. 5. Comparison of iCM contractility upon electrical stimulation on randomly oriented or aligned microfibrous scaffolds after 2 days of cell seeding

Cells were fluorescently labeled using calcein-AM (calcein) and corresponding videos of contractions (top left) after electrical stimulation (1 Hz) were then acquired. Contractility heat maps (top right) were generated using MATLAB. A representative histogram of beating speed to measure the periodicity of contraction is depicted (bottom). Comparisons are shown between (A) randomly oriented scaffold + iCM, (B) aligned scaffold + iCM, (C) randomly oriented scaffold + iCM + iEC, and (D) aligned scaffold + iCM + iEC. E. Quantification of maximum contraction velocity between groups ($n = 3$). Arrow denotes orientation of aligned microfibers. * denotes a statistically significant relationship ($P < 0.05$).

Fig. 6. Comparison of iCM contractility upon electrical stimulation on randomly oriented or aligned microfibrous scaffolds after 12 days of cell seeding

Contractility heat maps and representative histograms of beating speed are shown on (A) randomly oriented scaffold + iCM, (B) aligned scaffold + iCM, (C) randomly oriented scaffold + iCM + iEC, and (D) aligned scaffold + iCM + iEC. E. Quantification of maximum contraction velocity between groups. Arrow denotes orientation of aligned microfibers. * denotes a statistically significant relationship ($P < 0.05$, $n = 3$).

Table 1

Characterization of mechanical properties of the randomly oriented and aligned microfibrinous scaffold.

Characteristic	Randomly Oriented Scaffold (n)	Aligned Microfibrinous Scaffold (n)
Fiber diameter (μm)	14.3 ± 1.3 (n=3)	7.0 ± 0.5 (n=3)
Pore diameter (μm)	22.4 ± 2.1	18.9 ± 1.7
Porosity (% v/v)	34.7 ± 11.2	30.4 ± 2.8
Maximum tensile stress (MPa)	2.9 ± 0.1 (n=3)	10.2 ± 0.4 (n=3)
Elongation at break (%)	426.3 ± 44.0 (n=3)	82.2 ± 6.4 (n=3)
Young's modulus (MPa)	0.72 ± 0.05 (n=3)	1.81 ± 0.06 (n=3)