Corrections

APPLIED BIOLOGICAL SCIENCES

The authors note the following:
“The reported conduction velocity of our human cardiac micro-wires, in Table 1, should read 25.1 ± 7.7 cm/s, rather than 47.4 ± 12.4 cm/s. This error was due to an unaccounted scaling factor during microscope imaging. We acknowledge Dr. Nenad Bursac for notifying us of this error.


“The major conclusion of our manuscript, which is that we determine and use engineering design criteria for the formulation of cardiac microtissues from pluripotent stem cell-derived cardiomyocytes, is not impacted by this error.”

The corrected table appears below.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Conduction velocity, cm/s</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CMWs</td>
<td>25.1 ± 7.7</td>
<td>—</td>
</tr>
<tr>
<td>Healthy human heart</td>
<td>46.4 ± 2.7</td>
<td>[38]</td>
</tr>
<tr>
<td>Healthy human heart, Purkinje fibers</td>
<td>—200</td>
<td>[38]</td>
</tr>
<tr>
<td>Cardiomyopathic human heart during pacing</td>
<td>41 (min) – 87 (max)</td>
<td>[39]</td>
</tr>
<tr>
<td>Cardiomyopathic human heart during ventricular fibrillation</td>
<td>25 ± 4.0</td>
<td>[39]</td>
</tr>
</tbody>
</table>

Values of normal and pathophysiological conduction velocities of the human heart obtained from the literature are shown. Values for CMWs were measured from three separate experiments via optical-mapping techniques. Data are reported as the mean ± SEM.

BIOPHYSICS AND COMPUTATIONAL BIOLOGY

The authors note that the following statement should be added to the Acknowledgments: “Anton computer time was provided by the National Center for Multiscale Modeling of Biological Systems (MMBioS) through Grant P41GM103712-S1 from the National Institutes of Health and the Pittsburgh Supercomputing Center (PSC). The Anton machine at PSC was generously made available by D.E. Shaw Research.”

IMMUNOLOGY

The authors note that the author name Sami Zelka should instead appear as Sami Zelkha. The corrected author line appears below. The online version has been corrected.

Huaiping Yuan, Sami Zelkha, Marina Burkatovskaya, Rohit Gupte, Susan E. Leeman, and Salomon Amar

www.pnas.org/cgi/doi/10.1073/pnas.1420457111

Table 1. Comparison of conduction velocities of native human heart tissue and the CMW system

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Correction

APPLIED BIOLOGICAL SCIENCES

The authors note that the following statement should be added to the Acknowledgments: “We would also like to thank Fibralign for developing aligned collagen slides that were used in this study.”

www.pnas.org/cgi/doi/10.1073/pnas.1509023112
Design and formulation of functional pluripotent stem cell-derived cardiac microtissues

Nimalan Thavandiran, Nicole Dubois, Alexander Mikryukov, Stéphane Masse, Bogdan Beca, Craig A. Simmons, Vikram S. Deshpande, J. Patrick McGarry, Christopher S. Chen, Kumaraswamy Nanthakumar, Gordon M. Keller, Milica Radisic, and Peter W. Zandstra

Access to robust and information-rich human cardiac tissue models would accelerate drug-based strategies for treating heart disease. Despite significant effort, the generation of high-fidelity adult-like human cardiac tissue analogs remains challenging. We used computational modeling of tissue contraction and assembly mechanics in conjunction with microfabricated constraints to guide the design of aligned and functional 3D human pluripotent stem cell (hPSC)-derived cardiac microtissues that term cardiac microwires (CMWs). Miniaturization of the platform circumvented the need for tissue vascularization and enabled higher-throughput image-based analysis of CMW drug responsiveness. CMW tissue properties could be tuned using electromechanical stimuli and cell composition. Specifically, controlling self-assembly of 3D tissues in aligned collagen, and pacing with point stimulation electrodes, were found to promote cardiac maturation-associated gene expression and in vivo-like electrical signal propagation. Furthermore, screening a range of hPSC-derived cardionic condition box 5 (NKKX2.5) cardiomyocytes and 25% Cluster of Differentiation 90 OR (CD90)+ nonmyocytes optimized tissue remodeling dynamics and yielded enhanced structural and functional properties. Finally, we demonstrate the utility of the optimized platform in a tachycardic model of arrhythmogenesis, an aspect of cardiac electrophysiology, and the heart maintains homeostasis and, to a limited extent, repair in response to ischemic injury. In contrast with this native microenvironment, conventional in vitro model platforms for drug screening and toxicity testing use tissue culture-treated polystyrene surfaces coated with a basal membrane. These 2D substrates lack topographical cues, limit cell–ECM adhesion to only one side of the cell, and have an elastic modulus that is orders of magnitude greater than the native substrate of the targeted cell type. Additionally, cardiomyocytes in these assays are cultured either on their own, with conditioned media from stromal cells, or with a physiologically inappropriate proportion of supporting cell types. It is established that multiple cell types are required to build physiological tissue. Supporting cells, such as cardiac fibroblasts, provide mechanotransductive cues and paracrine factors that influence cardiomyocyte assembly and maturation. In addition, gradients of electrical and dynamic mechanical forces provide critical electro- and mechanotransduction signaling throughout development and maturation following disease, injury, and repair. Cell morphology, rate of proliferation, migration, differentiation potential, drug responsiveness, and juxtaphrone signaling are all influenced by ECM-mediated mechanotransduction. To accurately determine the input-cell composition on microtissue properties.

Directed differentiation strategies for generating and preserving human pluripotent stem cell (hPSC)-derived cardiomyocytes (CMs) are well-developed (1–7), and several CM cell-surface markers have been used to enrich target subpopulations have been discovered (8, 9). Although differentiation of hPSCs into contracting CMs is well-established, their maturation into adult-equivalent cells, and their formulation into functional adult-like tissue, remains an unmet challenge (5). We and others have previously developed in vitro heart tissue models (10–12); however, these recently developed systems require further definition in terms of their underlying design criteria and the impact of key design parameters such as tissue geometry and input-cell composition on microtissue properties.

In the developing heart, gradients of cytokines drive differentiation along various cardiovascular lineages and, later, the adult heart is capable of dynamically maintaining a balanced composition of cardiomyocytes, cardiac fibroblasts, smooth muscle cells, and endothelial cells within a highly ordered extracellular matrix (ECM). It is through complex cell–cell and cell–ECM interactions that the heart maintains homeostasis and, to a limited extent, repair in response to ischemic injury. In contrast with this native microenvironment, conventional in vitro model platforms for drug screening and toxicity testing use tissue culture-treated polystyrene surfaces coated with a basal membrane. These 2D substrates lack topographical cues, limit cell–ECM adhesion to only one side of the cell, and have an elastic modulus that is orders of magnitude greater than the native substrate of the targeted cell type. Additionally, cardiomyocytes in these assays are cultured either on their own, with conditioned media from stromal cells, or with a physiologically inappropriate proportion of supporting cell types. It is established that multiple cell types are required to build physiological tissue. Supporting cells, such as cardiac fibroblasts, provide mechanotransductive cues and paracrine factors that influence cardiomyocyte assembly and maturation. In addition, gradients of electrical and dynamic mechanical forces provide critical electro- and mechanotransduction signaling throughout development and maturation following disease, injury, and repair. Cell morphology, rate of proliferation, migration, differentiation potential, drug responsiveness, and juxtaphrone signaling are all influenced by ECM-mediated mechanotransduction. To accurately determine the input-cell composition on microtissue properties.

microfabrication | heart regeneration | tissue engineering | cardiac toxicity | arrhythmia disease model

Significance

Robust and predictive in vitro models of human cardiac tissue function could have transformative impact on our ability to test new drugs and understand cardiac disease. Despite significant effort, the generation of high-fidelity adult-like human cardiac tissue analogs remains challenging. In this paper, we systematically explore the design criteria for pluripotent stem cell-derived engineered cardiac tissue. Parameters such as biomechanical stress during tissue remodeling, input-cell composition, electrical stimulation, and tissue geometry are evaluated. Our results suggest that a specified combination of a 3D matrix-based microenvironment, uniaxial mechanical stress, and mixtures of cardiomyocytes and fibroblasts improves the performance and maturation state of in vivo engineered cardiac tissue.


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Frequently available online through the PNAS open access option.

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effects of test compounds, the niche of the target cell type—CMs in this case—must be recapitulated in an in vitro model. To engineer this niche, however, key design criteria must be established.

Engineered myocardial tissue models have been used to both elucidate fundamental features of myocardial biology and develop organotypic in vitro model systems for screening. Our previous engineered heart tissue design, which used centimeter-scale collagen scaffolds, had an isotropic matrix architecture (that induced limited CM alignment) and heterogeneity in spatial cell distribution within the volume of the matrix (10). Eschenhagen and co-workers have advanced the field by developing aligned tissue models suitable for some multiwell-plate analytics and that are capable of measuring forces of contraction (22). Together, these and other systems (23, 24) have already proven their utility as models for cell transplantation (25) and drug candidate evaluation (12, 26, 27). We have recently used microfabrication to miniaturize such aligned tissue models into microscale tissues (11). Despite these significant advances, we still do not have established criteria for the design and formulation of 3D microtissues, particularly with respect to input-cell composition, ECM–cell interactions during remodeling mediated by biomechanical forces, accessibility to rapid nondestructive measurements, tissue geometry and cell alignment properties, and, importantly, reproducible electrophysiological properties.

We describe an integrated computational and experimental strategy for the rational design of cardiac microtissues from cardiac differentiated hPSC derivatives. Our so-called cardiac microwire (CMW) system formulated NKX2-5+ cardiomyocytes and CD90+ nonmyocytes with electromechanical stimulation to generate microtissues that reproducibly mimic morphologic and functional properties of adult tissue. CMWs maintained densely aligned architectures and exhibited cell composition-specific remodeling, functional responsiveness to chemicals, and condition-specific gene expression patterns. In proof-of-principle studies, we demonstrated the utility of the platform in modeling tachycardia induced by arrhythmogenic cardiomyopathy, an aspect of cardiac electrophysiology not previously described using a cardiac microtissue model. The design criteria identified herein should accelerate the development of predictive in vitro assays of human heart tissue function.

Results

Finite-Element Modeling Predicts Stress-Mediated Assembly and Alignment of Cardiac Microtissues. Studies have repeatedly shown the impact of a 3D ECM microenvironment on the gene expression and functionality of contained cells, independent of cues from soluble factors. We reasoned that to engineer a more accurate heart cell niche, we would need to transition from 2D substrates to a 3D cell-encapsulating ECM geometry. To achieve this, we exploited the ability of single cells to remodel through their adhesion and contraction of the ECM. We engineered a versatile microfabricated platform and seeding protocol to increase the throughput of our studies (SI Appendix, Fig. S1). Although we were successful in generating cardiac microtissues, the geometries and seeding compositions were determined without precise design criteria and, as a result, the formed microtissues were variable and lacked robustness (SI Appendix, Fig. S2). Dissociated heart cells encapsulated in a gel will go through several phases: recovery of actin filaments and extension of filopodia, accumulation and assembly of cell-adhesion molecules that help with remodeling, increased expression of gap-junctional and contractile proteins, and finally excitation–contraction coupling, which permits the cardiac tissue to propagate action potentials and contract in unison (28). We hypothesized that by simulating the early remodeling process of gel compaction due to cell-contraction forces, we would gain insight into rational design criteria for generating cardiac microtissues.

To provide insight into the stress-mediated formation and contractility of sarcomeric filaments in cardiac microtissues, we developed finite-element (FE) models of microtissue and substrate geometries in our platform using a constitutive framework that accounts for the dynamic reorganization and contractility of the cytoskeleton (29). This framework has previously been implemented for the modeling of stress fiber contractility in a range of cell phenotypes (30–33) and for simulation of the response of cells to applied shear (34) and compression (35, 36), loading. Here it is adapted for the simulation of sarcomeric filaments in cardiomyocytes on the basis that both stress fibers and sarcomeric filaments are composed of and operate via actin–myosin interactions. Details of the constitutive model are provided in SI Appendix. Briefly, stabilized sarcomeres are predicted when the tension that is actively generated by sarcomeres is structurally supported in the microtissue. A reduction in tension in a given orientation results in a dissociation of actin–myosin contractile filaments in that direction, as illustrated in Fig. 1. As a first step, we explored in silico strategies to spatially predict stress distributions and sarcomere formation within complementary 3D tissue geometries, elucidating the relationship between the local microtissue stress state and sarcomere formation. Two microtissue geometries (Fig. 1) were simulated using our FE-based microtissue contractility model (29): (i) a square-shaped microtissue geometry designed to promote the generation of predominantly biaxial intratissue tension forces (BITFs), and (ii) a wire-shaped microtissue geometry designed to promote the generation of predominantly uniaxial intratissue tension forces (UNITFs).

Computational results are presented in terms of a non-dimensional effective uniaxial stress invariant $\langle S \rangle$ and in terms of the level of aligned sarcomere formation (II) (see SI Appendix for model output details). The predicted distribution and alignment of both quantities throughout the BITF and UNITF microtissues are shown in Fig. 2. Peripheral regions of the BITF microtissue experience a uniaxial stress state parallel to the...
microtissue boundaries (Fig. 2A, Left). In contrast, a biaxial stress state is predicted in the center of the BITF microtissues. As shown in Fig. 2B, the distribution and alignment of sarcomeres (H) corresponds closely to that of $S$. In the UNITF microtissue, a highly uniaxial stress state is generated throughout the longitudinal length, producing a uniform regime of uniaxial intratissue tension (Fig. 2B, Upper). A corresponding uniform distribution of highly aligned sarcomeres is also predicted in this region (Fig. 2B, Lower). In summary, aligned sarcomere formation is predicted in regions where the local stress state is uniaxial in nature (as $S \rightarrow 1$). In contrast, sarcomere structures are not computed in regions of the microtissue where the local stress state is biaxial in nature (as $S \rightarrow 0$). The computed evolution of the stress and sarcomere distributions throughout the microtissue geometries is presented in SI Appendix, Figs. S3–S7.

To empirically validate these predictions, we generated substrates as before, but now using the geometries specified in the computational models (SI Appendix, Fig. S8 A and B). We designed disk inserts containing the recessed arrayed poly(dimethylsiloxane) microwells and affixed them inside the bottom wells of a universal 24-well tissue-culture plate (SI Appendix, Fig. S8 C and D). We termed the UNITF microtissue the cardiac microwire. Rat neonatal heart cells were next used to test the system. We observed cells begin to extend filopodia and remodel the surrounding collagen matrix, and within 3 d the microtissues had formed and hit a plateau in morphology. Along with time in culture, we observed that higher concentrations of collagen prolonged the time and extent of microtissue remodeling, as did lowering the input-cell density. As predicted by our model, overall sarcomere expression in the BITF microtissue was observed to be spatially heterogeneous as revealed by immunostaining for cardiac sarcomeric proteins (Fig. 2C) in comparison with CMW (Fig. 2D). Also predicted by the model, regions exhibiting highly aligned sarcomeres correlated with areas of high stress. Morphologies of CMs in the BITF and CMW microtissues mirrored that of CMs on unaligned and aligned collagen substrates (SI Appendix, Fig. S9). In areas where our model predicted alignment due to high uniaxial stress, we observed elongated and oriented cell alignment parallel to modeled localized stress (Fig. 3 C and D). To test whether the induced alignment in the microtissues was similarly due to topographical cues (via the remodeled and aligned collagen fibrils), we probed the CMW after 3 d of remodeling using the LC-PolScope (Cambridge Research and Instrumentation (CRI)) quantitative birefringence imaging system. The color of the pixel in PolScope micrographs determines the orientation angle of the fibrillar collagen. We confirmed that the fibrillar collagen within the CMW was indeed remodeled and aligned in parallel to the longitudinal axis of the CMW (Fig. 2 D and E).

These results show that cardiac microtissues under uniaxial tension forces give rise to highly aligned tissues expressing spatially homogeneous contractile proteins that may better mimic cardiac muscle fibers in vitro. However, we were unable to explain the varying degrees of tissue organization (due to remodeling) observed with different input-cardiac cell numbers. To formally investigate this variability of assembly and phenotype in our tissues, we hypothesized that controlling cell composition may mitigate this effect.

**Input-Population Composition Impacts Tissue Morphogenesis and Cardiac Gene Expression.** To test the hypothesis that cell composition would affect tissue physiology, and to extend our analysis to the formulation of more translationally relevant human cardiac microtissues, we sorted hPSC-derived heart cells to generate tissues with specific input populations consisting of NKK2-5+/cardiomyocytes and CD90+ nonmyocytes (putatively fibroblasts; FBs). We applied our cardiac differentiation protocol to an NKK2-5-GFP reporter human embryonic stem cell (hESC) line that contained the EGFP cDNA inserted into the NKK2-5-GFP locus of HES3 hESC (1, 9). On day 20, we dissociated the hESC–CM aggregates and sorted for both of these populations to high purity (Fig. 4A). We generated CMWs of specific CM-to-FB
Aligned tissue architecture can be induced by controlling ECM topo-
graphy and intratissue mechanical stress. (A) Cell elongation of heart cells on
pseudo-3D–aligned and unaligned collagen substrates (Left) and cell
elongation of heart cells in CMWs (Right). Measurement of cell elongation is
the ratio of the major axis to the minor axis of a cell. (B) Cell orientation of
heart cells on pseudo-3D–aligned and unaligned collagen substrates (Left)
and cell orientation of heart cells in CMWs (Right). Measurement of cell
orientation on pseudo-3D substrates is relative to the direction of alignment
of patterned collagen. Measurement of cell orientation in CMWs is relative
to the direction of the longitudinal axis. (C) Fibrillar collagen content
of CMWs measured with the quantitative birefringent imaging system. Pixel
color corresponds to the angle of birefringent fibrillar collagen in
CMWs. (Insets) Higher-magnification images. (C) CMW held taut shows
unidirectionally aligned collagen. (D) Compacted CMW maintains fibrillar
collagen alignment in the direction of the curl. Data are reported as the
mean ± SEM. *P < 0.05 (Mann–Whitney U test).

ratios and controlled spherical reaggregates of the same ratios to
exclude effects due to dissociation, flow sorting, and reaggregation.
Conditions of the ratios 100:0, 75:25, 50:50, and 25:75 correspond,
respectively, to 100%, 75%, 50%, and 25% of NKX2-5-GFP+
cells, with the remainder consisting of CD90+ cells (Fig. 4B).
Both the CMW and reaggregate microtissues were cultured for
7 d. We observed familiar remodeling kinetics compared with our
previous experiments; however, there were clear differences in
tissue morphology and physiology between the tissue composition
conditions (SI Appendix, Table S1). ECM remodeling was ob-
served in all conditions; however, limited gel compaction was
observed in 100:0 CMWs, likely due to the lack of FB-associated
tissue remodeling (37). As the ratio of CD90+ cells increased, we
observed tighter, and more integrated, tissue morphologies under
higher apparent tension. CMWs formulated with ratios of 50:50
and 25:75 did not maintain tissue integrity to day 7 and failed due
to tension induced by fibrosis. Spontaneous contractions were
documented on day 7 for 100:0 and 75:25 CMWs, with limited
contractions observed in 50:50 CMWs. Synchronous contractions
were only observed in 75:25 and 50:50 CMWs; 100:0 CMWs
formed unstable tissue (in both the distal and medial locations)
with minimal cell–cell and cell–ECM integration and were un-
dergoing asynchronous contractions (Fig. 4C, Upper Left). These
CMWs contained nonintegrating globular aggregates of cells
separated by patches of collagen (SI Appendix, Movies S1 and S2).
Live-cell imaging of NKX2-5-GFP+ cells in the tissues confirmed
that the globular areas were 3D clusters of CMs that had either
proliferated into colonies or had conglomerated into clusters over
time (Fig. 4C, Lower Left). As the percentage of CD90+ cells
reached 25%, however, the CMWs took on a more robust archi-
tecture, with synchronous contractions resembling in vivo-like
tissue morphology (Fig. 4C, Right and SI Appendix, Movies S3
and S4). As a result, further electrophysiological measurements on
day 7, including conduction velocity, were amenable only in the
75:25 condition.

To determine the spatial localization of the FB population
within the tissues before and after tissue formation, we stained
and imaged for vimentin (intermediate filaments), a common FB
marker. FBs in nondissociated hESC–CM aggregates (Fig. 4D,
Insets and SI Appendix, Fig. S10A) displayed spatial heteroge-
neity and fibrotic-like areas within the tissue. In 75:25 CMWs,
however, FBs and CMs displayed spatial homogeneity, with
evenly dispersed marker expression (Fig. 4D and SI Appendix,
Fig. S10).

Aligned tissue and ultrastructure in the 75:25 CMWs, in com-
parison with aggregates of the same formulation (SI Appendix,
Fig. S11), demonstrate the benefits of uniaxial stress with respect
to cell spatial organization, apparent cell density, and cell align-
ment. It is noteworthy that tissue density is higher in CMWs
compared with aggregates. Additionally, despite seeding in
colagen, there is very little collagen present after the remodel-
ing phase in our CMWs, as evident by lack of collagen (blue) present
in our trichrome staining (SI Appendix, Fig. S11B). Ultrastructure
analysis using Transmission Electron Microscopy (TEM) dem-
onstrated increased length and alignment of the myofibrils and
sarcomeres, as well as the presence of Z disks and H zones in the
CMWs, relative to aggregate controls (SI Appendix, Fig. S12).

To study further effects of tissue formulation on tissue de-
development, we examined gene expression of key cardiac matu-
ration markers in our CMWs with respect to aggregates of
identical formulation after 7 d in culture. We first looked at
cardiac myocyte markers for determining dilution consis-
tency of input-cell composition. The NKX2-5 gene was the basis
of initial CM sorting, and so we used it as both a control and
a normalizing factor for measured cardiac-specific genes. As
expected, NKX2-5 expression showed a decreasing trend with
increasing dilution of CMs in the engineered tissue (Fig. 4E),
save for the 75:25 aggregate condition. This may perhaps be due
to an optimal balance of CM-supportive factors or cell–cell
contact (via CD90+ cells) in the matrix-free aggregates com-
pared with the encapsulated CMWs. DDR2, a marker for FBs,
showed a significantly increasing trend with decreasing dilution
of CD90+ cells. Cx43, expressed in both CMs and FBs, showed
consistently level trends. CM marker expression [Signal-regula-
tory protein α (SIRPA) and cTnT] in both aggregates and
CMWs also remained consistently level after being normalized
to NKX2-5 (Fig. 4F). By effectively controlling for CM number
by normalizing all CM-specific genes to NKX2-5, we diminished
the differences in CM control marker expression within the four
conditions. These results indicate that CMWs maintain their
initial dilution consistency, and that there are no significant
variations of control CM genes among conditions once tissue-
fraction CM number is controlled for.

We next examined the impact of tissue formulation and
composition on a panel of CM-specific genes indicative of CM
maturation. Markers of CM maturation (ANF, BNP, MYL2,
MYL7, MYH6, and MYH7) were also normalized to NKX2-5
expression levels to account for varying CM numbers in the
mixed population of tissue. Atrial natriuretic factor (ANF), secreted by the atria, and brain natriuretic peptide (BNP), secreted by the ventricle, are cardiac hormones that are involved in normal and diseased heart physiology. Although we did not see an increase of ANF expression in CMWs relative to the control aggregates, significantly increased BNP expression was observed in CMWs in both the 100:0 and 75:25 conditions relative to aggregates. Expression levels of MYL2 (MLC2v) and MYL7 (MLC2a), genes specific to sarcomere structure, were both also observed to be higher in the 75:25 CMWs. Although there were no significant differences in MYH6 (α-MHC) expression between conditions, MYH7 (β-MHC) expression was significantly higher for both the 75:25 and 50:50 conditions. The ratio of MYH7:MYH6, an indicative ratio of maturation, was found to be increased in CMWs for all conditions except the 100:0 condition (Fig. 4G). The up-regulation of these genes correlates with the conduction velocity of cardiac muscle, and may be due to the well-integrated and synchronously contracting tissues in the mixed-FB conditions.

It is evident from these gene expression data that there is an interactive effect between the biophysical microenvironment (aggregate versus CMW) and cell composition of tissue (ratio of CM to putative FB). The 75:25 CMW condition was optimal in terms of tissue morphology, and the CMW tissues (relative to the control aggregates) promoted more mature cardiac-specific gene expression. These findings suggest that the combination of a 3D matrix-based microenvironment under uniaxial mechanical stresses with a supporting cell-type fraction (25% CD90+ cells in this case) is a strategic focus in the design criteria of in vitro engineered tissues.
Electrophysiological Assessment of CMWs Indicates Functional Matura-
tion and Disposition to Modulation Using Electrical Stimulation. Our
results thus far indicate that tissue phenotype is shaped in part by
mechanical forces, input-cell composition, and the composition of
the extracellular matrix microenvironment. More specifically, we
determined that 75:25 microtissues gave rise to more archi-
tecturally robust phenotypes compared with other cell composi-
tions, and our CMWs provided a microenvironment that pro-
vided the increased expression of CM-specific maturation genes.
We next wanted to determine whether our 75:25 ratio could be used to study other cardiac tissue properties. Differentiated in-
put populations of CMWs were selected to fall within an ac-
ceptable range of 75:25 CMWs (75 ± 10% cTnT+) and were cultured for at least 7 d before being functionally assayed. We
focused on studying the excitability, resiliency to high-frequency
pacing, and conductivity of our 75:25 CMWs because these have
been previously shown to be indicative of electrophysiological
improvement in CMs. Excitation threshold and maximum cap-
ture rate of CMWs improved significantly compared with hESC–
CM aggregates (Fig. 5 A and B). Maximum capture rate improved
even further when CMWs were electrically point-stimulated with a
biphasic square wave pulse for 3 d. This was achieved by in-
tegrating the two flanking posts within the CMW microwell with
platinum wire electrodes to provide electrical point-stimulation
capability (SI Appendix, Fig. S8C). Point stimulation allows for
sequential activation of cells and promotes alignment of gap
junctions (which also occurs in normal heart maturation) as
opposed to field stimulation, which simultaneously stimulates all
cells in a tissue. We perturbed CMWs using drugs of known
effects and optically mapped their response [transmembrane
action potential (AP) and intracellular calcium transient] using voltage- and calcium-sensitive dyes, respectively. Addition of epi-
nephrine (0.1 μg/mL), an adrenergic neurotransmitter, to CMWs
increased the activation rate relative to the baseline, whereas
adding increasing concentrations of lidocaine (2 μg/mL), an anti-
arrhythmic drug, reduced and nearly abolished activation (Fig.
5C). Adding verapamil (0.25 μg/mL), an L-type Ca2+-channel
blocker, reduced the amplitude of calcium waves in CMWs relative to
the baseline; and supplementing with epinephrine increased the
rate of calcium transients (Fig. 5D). We also recorded conduction
velocities of CMWs and compared them with healthy (38) and
diseased (39) conduction velocities of the human heart (Table 1).
Remarkably, CMW conduction velocity (47.4 ± 12.4 cm/s) was found to be comparable to that of a healthy human heart (46.4 ±
2.7 cm/s).

As an extension, we studied and manipulated the dynamics of
activation propagation in CMWs. Typically, in the linear CMW
geometry, we observe a normal activation propagation initiate in
one of the distal ends, converge, traverse down the longitudinal
axis, diverge at the fork at the neck, and terminate at the distal
eend of the opposite tissue loop (Fig. 6 A and B). We next showed the ability to manipulate activation prop-
gagation directionality in CMWs using electrical point stimula-
tion. Starting with CMWs with spontaneous activation propa-
gation traversing from left to right (or right to left) (Fig. 6 B, C, and
D), we reversed the activation propagation direction by electrically pacing from the opposite end (Fig. 6 B, Lower and SI Appendix, Movie S7) in all test cases. When the point stimulus was removed, however, the propaga-
tion direction reverted to the original direction of spontaneous
propagation (SI Appendix, Table S2). In some cases of CMWs
presenting spontaneous electrical activity, however, we noticed a perturbation that led to unidirectional conduction block at the
junction of the medial and distal site (Fig. 6 C and SI Appendix, Movie S8). These unidirectional conduction blocks gave rise to incomplete looped activation propagation trajectories similar to
reentrant waves in arrhythmias caused by scar formation; however, they did not reenter the circuit and did not lead to
arrhythmia. We then extended our design to better model a re-
entrant wave in a tachycardic heart by manipulating the geo-
metry of our linear CMWs. CMWs were generated using a circular
template substrate to create a ring of tissue mimicking a re-
entrant wave during tachycardia around functionally unexcitable
scar tissue. Circular CMWs (CMWcirc) were seeded as previously
described and cultured for 14 d before assaying (SI Appendix, Movie S9). Reproducibility of CMWcirc remodeling was high
both within replicates (SI Appendix, Movie S10) and between separate experiments. Interestingly, the majority of CMWcirc
were observed to be in a reentrant state of arrhythmia after 14 d
of culture (82% ± 8%) (SI Appendix, Table S3 and Movie S11)
and the rest were in normal rhythms (SI Appendix, Movie S12). Elec
trophysiological assessment revealed spontaneous infinite
loop-like cycles of activation propagation traversing the ring as
expected (Fig. 6 D). Trace recordings indicate a high-frequency
activation rate with no rest period. Please see SI Appendix, Movie
S13 for a video of multiple cycles of activation propagation.
We next aimed to “defibrillate” the CMWcirc from a reentrant state
of arrhythmia to a normal rhythm state. Using carbon electrodes
spaced 1 cm apart, CMWcirc were field-stimulated at 10 V for 3 s.
After a 2- to 3-s period of inactivity, the CMWcirc, recovered to
a normal rhythm (~1 Hz) in all test cases (Fig. 6 E and SI
Appendix, Movie S14). Following culture of an additional 7 d, the
majority of CMWcirc remained in normal rhythm (95% ± 4%),
while a small percentage degenerated back to an arrhythmic
state (5% ± 4%). We have shown here that by simply modulating the
geometry of our tissue, we can recapitulate basic elements of a
disease model—in this case, tachycardia in a model of arrhyth-
mogenic cardiomyopathy.
Table 1. Comparison of conduction velocities of native human heart tissue and the CMW system

<table>
<thead>
<tr>
<th>Subject</th>
<th>Conduction velocity, cm/s</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CMWs</td>
<td>47.4 ± 12.4</td>
<td>—</td>
</tr>
<tr>
<td>Healthy human heart</td>
<td>46.4 ± 2.7</td>
<td>(38)</td>
</tr>
<tr>
<td>Healthy human heart, Purkinje fibers</td>
<td>−2,000</td>
<td>(38)</td>
</tr>
<tr>
<td>Cardiomyopathic human heart during pacing</td>
<td>41 (min)−87 (max)</td>
<td>(39)</td>
</tr>
<tr>
<td>Cardiomyopathic human heart during ventricular fibrillation</td>
<td>25 ± 4.0</td>
<td>(39)</td>
</tr>
</tbody>
</table>

Values of normal and pathophysiological conduction velocities of the human heart obtained from the literature are shown. Values for CMWs were measured from three separate experiments via optical-mapping techniques. Cardiomyocyte CMWs were found to exhibit conduction velocities on par with the epicardium of a healthy human heart. Data are reported as the mean ± SEM.

Discussion

Cardiac tissue engineering, whether for transplantation or in vitro modeling, requires key design criteria and processes for robust and reproducible results. The design methods and results presented here are, to our knowledge, a unique method of combining computational approaches with tissue design and “bottom-up” construction of hPSC-derived heart tissue using transcription- and surface marker-delineated heart cell populations. We demonstrate the importance of understanding and modulating (i) the biomechanical microenvironment and associated effects on spatial organization of structural and functional proteins during tissue growth in three dimensions, (ii) the cell composition of tissue and subsequent cell–cell and cell–ECM interactions, and, finally, (iii) the effect of geometry on tissue physiology. Previous studies proposing hPSC–CM–based in vitro models have not accounted for effects of intratissue stresses in the design phase with the aid of computational models simulating actin polymerization and myosin phosphorylation. Additionally, in previous studies, cell composition is usually not precisely engineered and, in the cases where input population was determined, none have studied the effects of ratio-based formulations of cocultured functional and stromal cell types.

In this study, we have adapted an FE model of tissue contractility via the active remodeling of stress fibers to describe the evolution and contractility of sarcomeric filaments in vitro. With this unique approach in determining design criteria for engineered tissue, we have demonstrated the importance of uniaxial stress-mediated sarcomere formation for building tissue expressing spatially homogeneous sarcomeric protein. Certainly insight from such in silico models, with the capability to recapitulate the organization and remodeling of the cytoskeleton, can have significant impact in the understanding and modulation of tissue morphogenesis in tissue-engineering applications.

Fig. 6. CMW activation propagation is disposed to directional modulation using electrical stimulation. (A) Activation propagation of normal CMWs. Each panel depicts a time lapse of the activation propagation along the longitudinal axis of the CMW. Phase-contrast image of CMWs, isochronal map, and timescale are indicated. (B) Direction of spontaneous activation propagation of normal CMWs (Upper) can be reversed using electrical point stimulation (Lower). (C) Activation propagation of CMWs observed to be obstructed by a conduction block, resulting in an incomplete reentrant wave-like system. (D) CMWs generated using a circular substrate (CMW*) designed to create a ring of tissue microring a reentrant wave during arrhythmia. Assessment revealed spontaneous infinite loop-like cycles of activation propagation traversing the ring; one cycle is shown. Signal tracings show multiple cycles. (E) Normal rhythm was observed in CMW* after defibrillation. An electrical field stimulation of 10 V was used to defibrillate arrhythmias in CMW* geometries to a normal rhythm. Signal tracings show multiple cycles. The initiation site in blue (T*) indicates the starting location of impulse propagation, and the termination site in blue (T†) indicates the final location of impulse propagation.
Importantly, results from our sorting studies highlight the importance of tissue composition due to improved functional assembly in CMWs composed of 75% NKK2.5+ and 25% CD90+ cells and improved mature cardiac gene expression in CMWs (relative to aggregates in select compositions). We report increased gene expression levels in CMWs of key cardiac maturation markers, including genes implicated in sarcomere structure, as well as cell-surface expression of markers that increase during the fetal heart gene program when organogenesis commences (40). We speculate that there may be high expression of integrins and adhesion proteins in the CD90+ cells that promote ECM remodeling and, as a direct result, bring cells together during tissue contraction to promote cell–cell contact, which has been shown to facilitate maturation signaling (41–43). Putatively fibroblasts, these CD90+ cells may also secrete growth factors such as bFGF and VEGF. Additionally, the remodeling may provide mechanotransductive cues such as tension forces, and in turn induce CM elongation and alignment.

In our optimal CMW system, we report conduction velocities on par with that of a healthy adult heart. We also show the capability of modulating activation propagation direction using electrical stimulation. Our ability to generate arrhythmia models, and then to defibrillate the arrhythmia to a normal rhythm, demonstrates the CMW system’s versatility and potential as a disease model and may serve as a platform to test the impact of antiarrhythmic drugs. Previous in vitro models of arrhythmogenesis do not exhibit the reproducibility and robustness of the CMW system, and additionally are not derived from hPSCs constructed in 3D tissue. Conversely, we plan to use the normal rhythm model as a tool to screen for proarrhythmic compounds.

The complete set of microenvironmental parameters of the myocardial niche was not recapitulated in our system, and so may likely explain our shortcomings of reaching some outputs from our CMWs that parallel that of native heart tissue. Indeed, there are other cell types and tissues not accounted for, including endothelial and immune cells in our system, along with their associated paracrine signaling. Nonetheless, our current model contains a basic subset of the elements needed to carry forward a sophisticated high-content screen of small molecules.

The versatility and customizability of the CMW platform allows for a variety of cell types and geometries to be generated en masse. For example, using the CMW geometry, noncardiac 3D tissue can be engineered where an initial stromal cell type, endothelial cells for instance, can be seeded and permitted to remodel. Next, a second functional cell type can be seeded circumferentially around the endothelium to create concentrically adjacent tissue types. Intertissue interactions between the adjacent tissue types can then be studied in a 3D tissue-like environment.

In conclusion, we demonstrate a bottom-up approach for the engineering of cardiac microtissues with the consideration of key design criteria. The robustness, flexibility, and increased throughput of our CMW platform highlight its potential as a powerful tool as an in vitro model for the screening of small molecules toward heart-regeneration therapies. Next-generation tissue-engineering approaches, to be effective, need to integrate these self-organization–based design criteria to build functional and reproducible tissue.

Materials and Methods

**Isolation of Rat Neonatal Cardiomyocytes.** Rat neonatal cardiomyocytes were isolated as previously reported (10). Briefly, hearts were isolated from 1- to 2-d-old neonatal Sprague–Dawley rats using protocol approved by the University of Toronto Committee on Animal Care. Rat hearts of one or two litters of ~13 pups per litter were aseptically excised and placed in cold HBSS (Sigma), washed several times with HBSS, and quartered. Quartered hearts were then incubated overnight at 4 °C in a 0.08% (wt/vol) solution of trypsin (Gibco) in HBSS on an orbital shaker at 0.5 g (Labent Orbit LS; Mandell). After 14–16 h, hearts were washed with CM culture medium [high-glucose (4.5 g/L) DMEM with -l-glutamine (Gibco) supplemented with 10% (vol/vol) FBS (Gibco), 1% (vol/vol) penicillin/streptomycin (Gibco), and 1% (vol/vol) Heps (Gibco)] and subjected to a series of five digests (8 min, 37 °C, 1 × g) in a 0.1% (wt/vol) solution of collagenase type II (Worthington) in HBSS. The supernatant of each digest was collected, centrifuged (5 min, 150 × g), and resuspended in CM medium. Cells were preplated for 60 min on tissue-culture polystyrene T75 flasks (BD Falcon) to enrich for cardiomyocytes (nonadherent cells). The supernatant was collected, and cell number was determined via trypan blue (Gibco) exclusion.

**Cardiac Differentiation of Human Embryonic Stem Cells.** Cardiac differentiation of human embryonic stem cells was carried out as reported previously (44). In this study, the HES2 (ES Cell International) HESC line was used. The hESCs were maintained and expanded as described previously. Briefly, HES2 cells were passaged (up to five times) on mouse embryonic feeders (MEFs) for 6 d in HES2 maintenance media (80% DMEM/F12, 20% KOSR, 20 ng/mL bFGF, 0.5% P/S, 1% NEAA, 1% BME); media were changed daily. Cells were maintained in normoxia at 37 °C in a 5.0% CO₂ atmosphere. The cells were then trypsinized along with MEFs and plated onto Matrigel (diluted 1:30)–coated plates at a split ratio of 1:3 for MEF depletion. After 2 d of MEF depletion, HES2 cells were again trypsinized and seeded into AggreWell Stemcell Technologies manufactured in-house to form human embryoid bodies (hEBs). The hEBs were generated using 400-μm microwell poly (dimethylsiloxane) (PDM5) inserts cast from a silicon master mold. The inserts were washed and glued into FlexCell tissue-culture inserts and then sterilized using ethanol. The microwells were then coated with 5% pluronic acid for at least an hour and washed with PBS before cell seeding. A single-cell suspension of aggregation media containing base media and T0 (day 0 of differentiation) cytokines supplemented with ROCK inhibitor Y-27632 was then seeded into the wells and allowed to aggregate overnight after centrifuging at 500 × g. Cells were maintained in normoxia at 37 °C in a 5.0% CO₂ and 5.0% O₂ atmosphere. After 24 h, hEBs were formed and aggregation media were exchanged for T1 media. On day 4, hEBs were removed from the AggreWells and placed in low-cluster six-well plates (Nunc). Corresponding media for T4, T8, and T12 were freshly made and exchanged. On T12, cells were returned to normoxia at 37 °C in a 5.0% CO₂ atmosphere. Media were replaced every 8 d onward.

**Microfabrication.** Masks for master patterning were designed using AutoCAD (Autodesk) and printed at a resolution of 20,000 dots per inch (CAD/Art Services). Microfluidic cell-culture devices were fabricated at the Emerging Communications Technology Institute cleanroom at the University of Toronto. Briefly, piranha-washed 3 × 5-inch clean glass slides (Corning) were given a brief wash in acetone and blow-dried under a clean stream of nitrogen gas. A seed layer of SU-8-50 (MicroChem) and 1 μm high was spin-coated onto the surface to allow for feature-layer bonding. Following a dehydration bake for 20 min on a 100 °C hot plate, the slides were then cooled to 65 °C and removed from the hot plate to return to room temperature. The seed layer was exposed to UV and postbake was initiated as before. The slides with the seed layer had two spin-coated layers of SU-8-50 (MicroChem) applied sequentially (including pre- and postbakes) to reach a feature height of 300 μm. The designed mask was UV-exposed onto the master with a 300-μm–high feature layer. Postbake, sufficient time was allowed for cooling. Immersion in developer (MicroChem) on a sonicator or orbital shaker was done until un–cross-linked SU-8 was washed away thoroughly. Masters were oven-baked for 3 d at 75 °C to allow proper bonding of the feature layer to the glass slide. Masters were rinsed in a desiccator overnight. Primary replicates were manufactured by molding PDMs (Dow Corning) on SU-8 masters at 65 °C overnight. Replicates were modified under a stereomicroscope, and a negative master was molded using polyurethane (SmoothCast). Final substrates were then PDMs-molded from these negative masters and outfitted onto a 24-well tissue-culture plate.

**Generation, Cultivation, and Imaging of Cardiac Microtissues.** Either rat neonatal CMs or hESC-derived CMs were suspended in a collagen master mix and seeded into cardiac microtissue wells at a density of 0.5 × 10⁶ cells per mL. Microwell substrates were prepared by stereilizing with ethanol and washing and coating with 5% (wt/vol) pluronic acid for at least an hour each. During the coating, rat neonatal CMs and/or hESC-derived CMs were prepared. Aggregates from hESC–CM differentiation were put in collagenase type II (1 mg/mL; Sigma) for 1 h with DNase in an incubator. Aggregates were then immersed in 0.25% trypsin for 5–10 min with DNase. Aggregates were then immersed in a STOP solution (2% BSA and 50% FBS) for an additional hour and rinsed with a 20-gauge syringe 10 times. Once aggregates were single cells, they were immersed in STAIN solution (10% FBS and 90% DMEM/F12) and counted. The collagen master mix was prepared by combining the following:

**Notes:**

1. PNAS PLUS | Published online November 18, 2013 | E4705

2. Thavandiran et al.
10× M199 (Gibco), Glutamax (Gibco), Collagen 1 (3.66 mg/mL) (BD Biosciences), glucose (0.3 g/mL) (Gibco), NaOH (Sigma), NaHCO₃ (0.075 g/mL) (Sigma), and dH₂O at appropriate ratios for the desired collagen concentrations. The collagen master mix was constantly kept on ice under 4 °C to prevent premature cross-linking. Finally, 500 μl of master mix was pipetted into each well (of a 24-well plate) and centrifuged at high speed (300 × g) to eliminate bubbles. The centrifuge was maintained at an ice-cold temperature. The cell-laden collagen (an additional 250 μl per well) was prepared and pipetted mixed into each well to a final cell density of 500,000 cells per well (the final volume in each well was 750 μl). The entire plate was centrifuged (200 × g) to force the cells into microwell recesses. Excess cell-laden collagen in each well was carefully and slowly aspirated to leave pockets of cell-laden collagen in each microwell. The entire plate was placed in a normoxic incubator for 15 min. After 15 min, 1 mL of cell-culture media was added slowly so as not to disrupt the polymerized collagen microspheres. Media were exchanged every 4 d. Microspheres remodeled between 1 and 3 d depending on input-cell composition. Imaging of microspheres was done in situ. Samples were fixed, permeabilized, and stained inside the microspheres and imaged using a fluorescence microscope.

Electrical Stimulation and Functional Analysis. For electrical point stimulation, microspheres were embedded with 0.005-inch–diameter platinum wires (99.99% purity; A-M Systems) and hooked up to a commercial stimulator (Grass S88X; Astro-Med). After 72 h of cultivation without electrical stimulation, the microspheres to be stimulated were stimulated with biphasic, square pulses 1 ms in duration, with a threshold amplitude of 6 V (field strength of 6 V/cm) and a frequency of 1 Hz for the remainder of cultivation (4 d). The stimulation voltage was selected to induce synchronous construct contractile rhythms. Constructs were held in place within the PDMS substrate.

Tissue function was established by measuring excitation threshold (ET), the minimum voltage required to pace the tissue simultaneously, and maximum capture rate (MCR), the maximum stimulation rate at which the construct can be induced to beat simultaneously, at 7 d after cell seeding. Tissue constructs or CM aggregates were individually placed between a pair of carbon electrodes in stimulation chambers (autoclaved before use). ET (V/cm) was measured by stimulating the tissue with square pulses of 2-ms pulse width at a frequency of 1 or 2 Hz and gradually increasing the output voltage of the stimulator until >80% of the tissue was being beating synchronously with the stimulator output. MCR was measured by setting the output voltage at 12 V and increasing frequency until >80% of the tissue was no longer synchronously beating with the driving signal. All measurements were taken using an Olympus TX2-UCB inverted fluorescence microscope housed in an environmental chamber (Solent Scientific) maintained at a temperature of 37 °C and equipped with a Retiga camera (QImaging).

Flow Cytometry and Cell Sorting. For flow cytometric analysis, aggregates were dissociated using collagenase treatment and trypsin and immediately fixed with 4% paraformaldehyde (PFA) overnight at 4 °C. They were then permeabilized and stored at room temperature with 100% methanol for 2 min. Primary antibody was added after a 2% PFA wash. It was then incubated at room temperature for 20 min. Next, the sample was washed with HF and the secondary antibody was added for another 20 min at room temperature. Last, the sample was washed again to be ready for flow cytometric analysis. The samples were always kept on ice before measurement by flow cytometer.

For flow cytometric cell sorting, EBs were dissociated as previously described (45). Cells were stained with anti-CD90 allophycocyanin (BD PharMingen; 1:500). Stainings were carried out in PBS with 10% FCS on ice. The cells were sorted using a FACSAria TMII (BD Biosciences) cell sorter (SickKids-University Health Network Flow Cytometry Facility). Data were analyzed using FlowJo software (Tree Star).

Immunostaining and Image Analysis. Microspheres were washed with PBS and fixed for 24 h with 4% PFA at 4 °C. They were then permeabilized in 0.1% Triton X in blocking solution (normal donkey serum). Primary antibody was then added for 3 d at 4 °C. Last, the microspheres were washed three times and stained with the appropriate secondary antibody (Alexa Fluor series) and with DAPI for nuclear staining overnight at 4 °C. Each incubation step was preferably performed on a rocker table. Before imaging, the sample was washed three times and resuspended in 2% HF. Samples were imaged using confocal microscopy (FV1000 laser scanning confocal microscope; Olympus). All image analysis was done using custom macros built in Image (National Institutes of Health) (cell alignment and elongation analysis, and total cell-marker expression enumeration).

Quantitative Real-Time PCR. Total RNA was prepared with the RNea¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬‐ 16 × 107} cells × ml{−1} (32–33). Dye fluorescence was recorded using a confocal microscopy system (Ultima; SciMedia). The system included a CMOS camera with a 1-cm sensor (100 × 100 pixels) attached to a custom-built microscope using a Plan Apo objective and condensing lenses (Leica Microsystems), giving a magnification of 1.5x. The spatial resolution was 63 μm per pixel. The fluorescence was excited using a xenon light source (Moticet) and a 405-nm green filter (Semrock), and the emission signal was low-pass filtered using a 610-nm red filter. Tissue constructs were point-stimulated at a 1,000-ms cycle length using a bipolar electrode made with two fine silver wires ([American Wire Gauge 32; A-M Systems Inc.] 32) inserted into a large stainless steel needle mounted on a micromanipulator. Spontaneous tissue beating was also recorded, in addition to responses to responses sweep from 1 to 5 Hz. Local activation times were measured at the peak of di/dt (dV/df) of fluorescence of fluorescent dye. Activation maps were con- structed for a selected beat. Conduction velocity was calculated at each location using activation times of nine neighboring sites. Conduction velocity values from all sites were used to calculate the average conduction velocity across the construct surface; minimum and maximum values were also noted. Phase-contrast images of microsphere tissues were taken before optical mapping to correlate tissue architecture geometry with conduction velocity.

Statistical Analysis and Data Representation. Statistical significance was computed using the Mann-Whitney U test. All error bars represent the SEM of three or more biological replicates. Asterisks indicate statistical significance between conditions of P < 0.05. All data analyses, including graphical representations, were performed using Excel (Microsoft); statistical analysis was performed using custom macros written in R programming language (R Development).

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