Myocardial Tissue Engineering With Cells Derived From Human-Induced Pluripotent Stem Cells and a Native-Like, High-Resolution, 3-Dimensionally Printed Scaffold

Ling Gao, Molly E. Kupfer, Jangwook P. Jung, Libang Yang, Patrick Zhang, Yong Da Sie, Quyen Tran, Visar Ajeti, Brian T. Freeman, Vladimir G. Fast, Paul J. Campagnola, Brenda M. Ogle, Jianyi Zhang

Rationale: Conventional 3-dimensional (3D) printing techniques cannot produce structures of the size at which individual cells interact.

Objective: Here, we used multiphoton-excited 3D printing to generate a native-like extracellular matrix scaffold with submicron resolution and then seeded the scaffold with cardiomyocytes, smooth muscle cells, and endothelial cells that had been differentiated from human-induced pluripotent stem cells to generate a human-induced pluripotent stem cell–derived cardiac muscle patch (hCMP), which was subsequently evaluated in a murine model of myocardial infarction.

Methods and Results: The scaffold was seeded with ~50,000 human-induced pluripotent stem cell–derived cardiomyocytes, smooth muscle cells, and endothelial cells (in a 2:1:1 ratio) to generate the hCMP, which began generating calcium transients and beating synchronously within 1 day of seeding; the speeds of contraction and relaxation and the peak amplitudes of the calcium transients increased significantly over the next 7 days. When tested in mice with surgically induced myocardial infarction, measurements of cardiac function, infarct size, apoptosis, both vascular and arteriole density, and cell proliferation at week 4 after treatment were significantly better in animals treated with the hCMPs than in animals treated with cell-free scaffolds, and the rate of cell engraftment in hCMP-treated animals was 24.5% at week 1 and 11.2% at week 4.

Conclusions: Thus, the novel multiphoton-excited 3D printing technique produces extracellular matrix–based scaffolds with exceptional resolution and fidelity, and hCMPs fabricated with these scaffolds may significantly improve recovery from ischemic myocardial injury. (Circ Res. 2017;120:1318-1325. DOI: 10.1161/CIRCRESAHA.116.310277.)

Key Words: apoptosis ■ cardiomyocyte ■ endothelial cells ■ heart ■ myocardial infarction ■ tissue engineering
What is Known?
• Cellular therapy for myocardial repair has shown some benefit in preclinical and clinical settings, but limited cell retention and associated lack of robust electromechanical coupling remain the major problems.
• Greater benefits may be achieved if the transplanted cardiac cells are provided with structural support such that synchronized contractile activity of the graft can be integrated to recipient heart.
• One approach to generate a structural support is 3D printing, which has recently advanced to include the printing of biological materials such as cells and extracellular matrix proteins.

What New Information Does This Article Contribute?
• The article describes the implementation of multiphoton-excited 3-dimensional printing to generate a scaffold with native-like cardiac extracellular matrix architecture. The scaffold can effectively harbor stem cell–derived cardiac cell types and can be handled for effective transfer to the damaged heart.
• The cell-seeded scaffold (termed human cardiac muscle patch) beats synchronously in a culture dish before transplant and with extended culture time beats with increased speed and calcium handling activity of the graft can be integrated to recipient heart.

Novelty and Significance

We demonstrate multiphoton-excited 3D printing to generate a human cardiac muscle patch that could be effectively populated with cardiac cell types because it mimics the structural dimensions and protein composition of the native myocardium. In a mouse model of myocardial infarction, human cardiac muscle patch transplantation results in higher levels of cell engraftment, cell proliferation, and myocardial vascular density, which are accompanied by a significant reduction of infarct size and improvement of left ventricular function. This work pushes the technical limits of 3D printing for cardiac tissue equivalents such that cells and extracellular matrix proteins could be manufactured precisely as designed.

Methods
A detailed description of the experimental procedures used in this investigation is provided in the Online Data Supplement.

Results
Fabrication of an ECM Scaffold Based on Templates Derived From Optical Image Stacks of Murine Myocardium
Our approach can be summarized in 2 steps: first, native, murine, adult myocardial tissue was examined to determine the size and distribution of various ECM features, which were incorporated into a 3D template; then, the template was scanned and used to map the positions of crosslinks in a solution of a photoactive polymer (Figure 1A). Importantly, our scanning technique, modulated raster scanning, maps the template directly to the scaffold by monitoring the brightness of each point in the image, and it is accurate to the resolutions of <1 μm. To our knowledge, this is the first time modulated raster scanning has ever been successfully used to control the fabrication of a tissue-engineered scaffold, and, consequently, our results are particularly relevant for applications that require the fibrillar and mesh-like structures present in cardiac tissue.¹⁸

We chose to base our template on the distribution of fibronectin in murine myocardium (Figure 1B and 1C). Fibronectin is uniformly distributed around each cardiomyocyte, so it can be used to determine the dimensions of each individual cell

cardiovascular tissues. However, key structural features of the native ECM need to be identified and incorporated (with adequate resolution and reliability) into a scaffold to promote cell function and limit detrimental effects. In addition, from the potential clinical application perspective, a high level of quality control of the scaffold product is required, which can only be achieved by a computer-controlled 3D printing technology.

The position of crosslinks within a matrix of photoactive biological polymers can be controlled with high resolution. Printers that use single-photon excitation coupled to a sequence of photomasks can achieve ≈30 μm resolution in x and y and ≈50 μm resolution in z. A more advanced technique, multiphoton-excited (MPE) photochemistry, can restrict excitation (and, consequently, the photochemical reaction) in 3 dimensions via a method that is analogous to multiphoton laser scanning microscopy.⁵–¹⁴ Notably, the resolution of the features (<1 μm) is determined by the MPE point spread function and can therefore approximate the feature size of components of the ECM.¹⁵ The technique can also be combined with rapid prototyping and computer-aided design⁶ to fabricate essentially any 3D structure that can be drawn.

For the experiments described in this report, we used our novel technique, MPE 3-dimensional (3D) printing, to generate a scaffold with a native-like cardiac ECM architecture from a solution of a photoactive gelatin polymer and then seeded the scaffold with cardiac cells (cardiomyocytes, endothelial cells [ECs], and smooth muscle cells [SMCs]) that had been differentiated from human cardiac-lineage–induced pluripotent stem cells (hCiPSCs)¹⁷ to generate an hCiPSC-derived cardiac muscle patch (hCMP). The hCMP was subsequently characterized via a series of in vitro analyses and then tested in a murine model of ischemic myocardial injury.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>3D</th>
<th>3-dimensional</th>
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<tr>
<td>ECs</td>
<td>endothelial cells</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>hCiPSC</td>
<td>human cardiac-lineage–induced pluripotent stem cell</td>
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<td>hCMP</td>
<td>human-induced pluripotent stem cell–derived cardiac muscle patch</td>
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<td>hiPSCs</td>
<td>human-induced pluripotent stem cells</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>MPE</td>
<td>multiphoton excited</td>
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<td>SMCs</td>
<td>smooth muscle cells</td>
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compartment to form a grid (here termed, adult simulate). The scaffold was generated from a solution of gelatin methacrylate, which can be crosslinked into complex structures with high efficiency, allows creation of complex structures with thickness of $\approx 100 \mu m$ (Online Movies I through III), and is biologically inert when both crosslinked and degraded, and the denatured collagen exposes cell binding sites (including Arg-Gly-Asp [RGD]), which should readily adhere to the seeded cells and support biochemical signaling via focal adhesions. Analysis of the native myocardium suggested that cardiomyocytes reside in channels that are $\approx 15 \mu m$ by $100 \mu m$ which, when incorporated in the hCMP scaffold (Figure 1D and 1E), yielded a robust structure with high reproducibility and exceptional fidelity in both coverage area ($\geq 95\%$) and intensity variation ($\geq 85\%$).

Integration of hciPSC-Derived Cardiac Cells in hCMPs

The hciPSCs were reprogrammed from human cardiac fibroblasts and differentiated into hciPSC-cardiomyocytes, hciPSC-SMCs, and hciPSC-ECs; then, the differentiated cells were characterized via the expression of lineage-specific markers (Online Figure I), and the hCMP was formed by seeding $\approx 50000$ cells (in a 2:1:1 ratio of hciPSC-cardiomyocytes, hciPSC-SMCs, and hciPSC-ECs, respectively) into the fabricated scaffold, where they quickly became assimilated and occupied most of the free space (Figure 1D). The hciPSC-cardiomyocytes began generating calcium transients (Figure 2A) and beat synchronously across the entire hCMP within 1 day of seeding (Online Movies IV and V). Over the next 7 days, the speeds of contraction and relaxation, the peak amplitude of the calcium transients (Figure 2B and 2C), and the expression of several genes required for contractile function (cTnT [cardiac troponin T], cTnI [cardiac troponin I], and $\alpha$-MHC [alpha myosin heavy chain]) and for generating calcium transients (SERCA2$\alpha$ [sarco/endoplasmic reticulum Ca$^{2+}$-ATPase], RYR2 [ryanodine receptor 2], and CASQ2 [calsequestrin 2]) increased significantly (Online Figure II). Contractile and calcium-transient gene expression was also greater in the hCMPs than in monolayers grown from equivalent populations of hiPSC-derived cardiac cells on day 7. Optical mapping of transmembrane potentials (Figure 2D and 2E) revealed macroscopically continuous action potential propagation in hCMPs, showing an excellent functional electrophysiological
communications between cells. Conduction velocity in hCMPs increased linearly with the increase of pacing cycle length, reaching 18.8±0.8 cm/s at 800 ms pacing cycle length (Figure 2F). Action potential durations, APD_{50} and APD_{80}, were ≈214±10.4 and 270±12.7 ms at 800 ms pacing cycle length, separately, and also showed significant correlation with pacing rate (Figure 2G). Autofluorescence images obtained via 2-photon microscopy on day 7 (Figure 2H) indicated that the cells had aligned with the channels of the fabricated scaffold (Figure 2I) to form multinucleate cells with an aspect ratio (ie, cell length:width) of ≈5.5:1 (Figure 2J), which is close to the characteristic ratio observed in native cardiomyocytes (7:1). Analysis of hCMPs stained for the expression of the cardiomyocyte protein cTnI, α-smooth muscle actin, and the endothelial marker CD31 (Figure 2K) indicated that the original 2:1:1 ratio of hciPSC-cardiomyocytes, hciPSC-SMCs, and hciPSC-ECs was largely retained (Figure 2 L), with cardiomyocytes comprising ≈45% of the remaining cells. The 7-day survival rate of hciPSC-cardiomyocytes in the hCMP was similar to that observed when cells were maintained on Matrigel-coated plates and significantly greater than the rate achieved by culturing the cells with bovine pericardium or polyethylene glycol (Figure 2M).

In Vivo Evaluations of hCMP Transplantation in a Murine Model of Myocardial Infarction

In vivo assessments of the hCMPs were performed in a murine model of myocardial infarction (MI). MI was surgically induced as described previously,20 then animals in the

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Figure 2. In vitro assessments of the human-induced pluripotent stem cell–derived cardiac muscle patch (hCMP). A, Calcium transients were recorded in hCMPs on d 1, 3, and 7 after cell seeding and used to calculate the (B) peak amplitude (F/F_0; n≥50 cells per time point). C, Videos of the beating hCMPs were taken on d 1, 3, and 7 and evaluated with motion vector analysis software to calculate the speeds of contraction and relaxation (n=4, hCMPs per time point). D–G Action potential propagation in hCMP was measured on d 7 (n=4). D, Representative isochronal map of activation spread and (E) selected optical V_{m} traces recorded during pacing with cycle length (CL) of 800 and 300 ms. Dependence of (F) conduction velocity (CV) and (G) action potential duration (APD_{50} and APD_{80}) on pacing CL. H, hCMPs were stained with DAPI on d 7; then, autofluorescence images were obtained via 2-photon microscopy (scale bar=20 μm) and used to calculate the cells’ (I) angle of alignment relative to the long axis of the engineered channel and (J) aspect ratio. K, On d 7, endothelial cells (ECs), smooth muscle cells (SMCs), and cardiomyocytes (CMs) were identified in the hCMPs via immunofluorescence staining for the presence of CD31, α-smooth muscle actin (α-SMA), and cardiac troponin I (cTnI), respectively; then (L) the proportion of each cell type was calculated (5 fields per hCMP and 4 hCMPs). M, Known quantities of human-induced pluripotent stem cells (hiPSC)-CMs were seeded into Matrigel, polyethylene glycol (PEG), decellularized bovine pericardium, or the engineered scaffold and cultured for 7 d; then, the cells were immunofluorescently stained for cTnI expression, and cell survival was quantified as the ratio of the number of cTnI+ cells observed to the number of seeded cells. **P<0.01.
MI+hCMP group were treated with 2 hCMPS, animals in the MI+Scaffold group were treated with 2 patches of scaffold material without cells, and animals in the MI group received neither the hCMP nor the cell-free scaffold. The hCMPS and scaffolds were positioned over the site of infarction (Figure 3A) and held in place by covering them with a piece of de-cellularized bovine pericardium that was sutured to the heart to avoid movement of hCMPS off the heart. The de-cellularized bovine pericardium above the hCMP has beneficial effect of preventing the adhesion between the heart/hCMP and the chest. The Sham group underwent all the surgical procedures for MI induction except the ligation step and recovered without either experimental treatment. Quantitative polymerase chain reaction measurements for expression of the human Y chromosome indicated that 24.5±2.6% of the transplanted cells remained engrafted in the hearts of MI+hCMP animals for at least 1 week after transplantation (Figure 3B). By week 4, the engraftment rate had declined to 11.2±2.3%, which is still much higher than the rate reported in previous studies of cell-based myocardial therapy.21,22 To assess the engraftment rate by counting the grafted human cells as evidenced by human-specific nuclear antigen positivity, we also used histological assessment of consecutive sections of the heart that spans the entire area covered by the engrafted patch and counting the grafted human cells as evidenced by human-specific nuclear antigen positivity.17,23 The engraftment rate of the 7 hearts studied using the histology method is 13.6±2.2%, which is in agreement with the quantitative polymerase chain reaction (11.2±2.3%). The ratio of the hCiPSC-tri-lineage cardiac cells at weeks 1 and 4 was also examined. The engrafted hCiPSC-cardiomyocytes were identified by the expression of both green fluorescent protein and cTnI, engrafted hiPSC-ECs were identified by the expression of human-specific CD31, and engrafted hiPSC-SMCs were identified by the expression of both green fluorescent protein and α-smooth muscle actin.

At week 1 post-transplantation, the ratio of hCiPSC-cardiomyocytes, hCiPSC-SMCs, and hCiPSC-ECs was 1.42:0.93:1; at week 4, the ratio was 0.67:0.86:1 for hCiPSC-cardiomyocytes, hCiPSC-SMCs, and hCiPSC-ECs, respectively. Hematoxylin and eosin staining (Figure 3C) and immunofluorescence analysis of human-specific nuclear antigen, green fluorescent protein, cTnI, α-smooth muscle actin, and human-specific CD31 expression (Figure 3C and 3D) showed evidence of all 3 transplanted cell lineages in the treated region.

Cardiac function was evaluated on day 28 after injury via echocardiographic assessments (Figure 4A) of left ventricular ejection fraction and fractional shortening; both parameters were significantly greater for animals in the MI+hCMP group than in MI+Scaffold or MI animals (Figure 4B and 4C), although measurements in the MI+Scaffold and MI groups were similar. Infarcts were also significantly smaller, although the thickness of the infarcted region of the myocardial wall was significantly greater in MI+hCMP hearts than in MI or MI+Scaffold hearts (Figure 4D through 4F), and analyses of TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling)-stained sections (Online Figure IIIA and IIIB) and sections stained for the presence of CD31 and α-smooth muscle actin (Online Figure IIIC through IIIE) indicated that apoptotic cells were significantly less common, whereas vascular structures (including arterioles) were significantly more common, at the border of the infarcted region in hCMP-treated animals than in the corresponding regions of hearts from animals in the MI+Scaffold or MI groups. hCMP treatment was also associated with significant increases in the number of cells...
that expressed the proliferation marker Ki67 (Online Figure IV). Thus, the transplanted hCMPs seemed to improve recovery from myocardial injury by, at least in part, reducing apoptosis, promoting angiogenesis, and increasing cell proliferation.

**Discussion**

The fate specification of progenitor cells in the developing heart is critically dependent on the spatial and temporal factors of the ECM where the progenitor cells reside. In human pluripotent stem cell differentiation to ECs, the rate and quality of derived ECs are critically influenced by the temporal factors in 3D ECM. The myocardial ECM provides substrates for cell adhesion, sequesters soluble factors, and serves as a conduit for mechanical signaling. Our recent efforts to characterize the ECM of the developing heart have provided us with an extensive body of knowledge on the nanoscale distribution of the ECM, and by combining this knowledge with MPE 3D printing, we were able to generate a scaffold that is structurally native like. The technique couples MPE with modulated raster scanning to induce crosslinks in a solution of a photoactive gelatin polymer, thereby creating an ECM scaffold with exceptionally high resolution. Notably, construction was guided by a template composed of features that had been identified in the ECM of a native adult murine heart, which suggests that the technique may be able to replicate the unique architecture of the myocardial ECM in each individual. Furthermore, when hCMPs were generated by seeding the scaffolds with hciPSC-derived cardiac cells and transplanted into infarcted mouse hearts, the treatment was associated with significant improvements in cardiac function, infarct size, apoptosis, vascular density, and cell proliferation. Thus, the hCMPs produced for this report may represent an important step toward the clinical use of 3D printing technology.
Our novel technique is the first to achieve a resolution of $\leq 1 \mu m$ and, consequently, can reliably control the thickness of the walls that separate adjacent channels. Channel wall thickness may be a particularly important parameter for myocardial tissues because contractions in adjacent fibers must be synchronized by signaling mechanisms that traverse the wall. Notably, the seeded cells quickly settled into the engineered channels of the scaffolds generated for this report, and synchronized beating was observed as early as 1 day after cell seeding, which suggests that individual cells interacted with the features of the scaffold, and that interchannel coupling mechanisms were quickly established.

The functional improvement associated with hCMP transplantation in the murine MI model suggests that this goal may have been at least partially achieved. Thus, in future investigations, we will use optical mapping technology to determine whether transplanted hCMPs are electromechanically coupled to native myocardial tissues.26

In conclusion, we have developed a method by which the principles of 3D printing and photochemistry can be combined to generate an ECM scaffold with unprecedented resolution from a template based on the architecture of native myocardial ECM. When hCMPs were generated by seeding the scaffolds with hciPSC-derived cardiac cells, the scaffold promoted cell viability and electromechanical coupling in vitro, and the hCMPs were associated with high levels of cell engraftment, as well as significant improvements in cardiac function, infarct size, apoptosis, vascularity, and cell proliferation in a murine MI model. Subsequent investigations will focus on methods for creating hCMPs of sufficient size for large animal studies and means to improve effectiveness of the hCMPs by incorporating mixtures of ECM proteins into the scaffold.

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**Disclosures**

None.

**References**

1325

Gao et al 3D Printed Scaffolds for Engineered Myocardium


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Supplemental Material

Myocardial tissue engineering with cardiac cells derived from human induced-pluripotent stem cells and a native-like, high-resolution, 3-dimensionally printed scaffold

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Short title: Gao, 3D-printed scaffolds for engineered myocardium

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SUPPLEMENTARY METHODS

Creation of the ECM scaffold via 3D-MPE printing

Methacrylated gelatin (100 mg/mL) was mixed with sodium 4-[2-(4-morpholino)benzoyl-2-dimethylamino]-butylbenzenesulfonate (MBS) (1 mM) at 1% v/v, and crosslinking was performed on a glass slide, which served as the non-specific background. Two-photon excitation of the MBS photoactivator was induced with a 100 fs Ti:sapphire laser (Mira, Coherent, Santa Clara, CA) at 780 nm. A 10x, 0.5 NA objective lens was used, and the average power at the focus was kept constant (~100 mW at 78 MHz repetition rate). Upon UV absorption, MBS degrades to benzoyl and an α-aminoalkyl radical, which then attack residues containing aromatic groups and free amines; then, the radical protein links to a second protein molecule, generating a covalent bond.

Our custom-built multiphoton instrument, which has been described in detail previously, can produce scaffolds with more complexity and in a greater variety of sizes than can be achieved with a commercial laser-scanning microscope. The Ti:sapphire laser is coupled to an upright microscope stand (Axioskop 2, Zeiss, Thornwood, NY), and scanning is performed by combining a laser-scanning galvos (Cambridge Technologies, Bedford, MA) with a motorized stage (x-y-z, Ludl Electronic Products Ltd, Hawthorne, NY). The apparatus is controlled with LabVIEW software, and data is acquired with a field-programmable gate array (FPGA) board (Virtex-II PCI-7831R, National Instruments, Austin, TX). The laser power entering the optical train is regulated with a 10 kHz electro-optic modulator (EOM) (Conoptics, Danbury, CT), and the laser is rapidly shuttered with a second, higher-speed EOM (maximum 100 MHz; Conoptics). Parameters such as power, scanning area, the scan rate of the galvanometer, and repetition of the scanning pattern (i.e., the number of scans per layer) are set via a graphical user interface (GUI), while the TPEF of the entrapped residual photoactivator
serves as the online diagnostic signal for crosslinking and is read by the FPGA. The microscope is also equipped with phase-contrast and two-photon fluorescence imaging capabilities for characterizing the scaffold (e.g. immunofluorescence and quality control), and the minimum size of the features in the crosslinked protein structure corresponds to the two-photon excited point spread function (PSF). For example, when using 0.75 NA and 780 nm two-photon excitation, the lateral and axial resolution are about 600 nm and 1.8 μm, respectively. MPE polymerization can also produce features with sub-micron resolution, because of chemical nonlinearity in the free radical kinetics, but this does not occur during the crosslinking of proteins.

The FPGA was incorporated into the system to exploit the parallelism of command executions (80 MHz clock rate) and to avoid bottlenecks in communication between the CPU and the hardware, which occurs through four first in, first out (FIFO) channels. Two FIFO channels relay information from the main LabVIEW program to the FPGA for control of the galvanometer mirrors and fast EOM shutter, while the other two record information from the PMT to create an image of the fabrication; thus, communication between the CPU and hardware occurs in near real-time. The source code of the instrument control software is freely available at: http://campagnola.molbio.wisc.edu/.

The scanning approach (i.e., “modulated raster scanning”) combines the advantages of both raster scanning and vector scanning. The galvanometers are raster scanned at maximum speed (~40 kHz), while the laser is shuttered at an even greater rate (10 MHz-40kHz) with the fast EOM. This approach is more accurate than modulating the scanning speed and/or step size of the galvanometers, because it is run over the whole field of view at constant speed and with the same pulse energy and repetition rate. The average power entering the fast-shuttering EOM is set by the slower EOM, which allows the entire scanning process to be performed at constant peak power and, consequently,
the fractional “on-time” within each pixel defines the integrated exposure dose, which is a linear process that directly correlates to the resulting protein concentration. Thus, we achieve a linear map between the intensity in the original image data and the fabricated structure.

Fabrication was based on 8-bit, 3D image files (.bmp or .tif) that were acquired with sampling that satisfied the Nyquist criterion, which is crucial for ensuring that the tissue is accurately represented and that the resolution (i.e., the sizes of the features) matches that of the original image, as well as for optimizing the structural integrity of the fabricated scaffold in 3D. Fabrication proceeded through the image stack via a Z-step pattern, and upon completion, the fidelity of the structure to the image stack was measured by using FIJI software to compare, pixel-by-pixel, the gray-scale intensity of the original (or processed) image to the two-photon excited fluorescence image of the fabricated construct.

**Generation and characterization of hciPSC-derived cardiac cells**

Recent reports from our laboratory suggest that the cells derived from induced-pluripotent stem cells (iPSCs) may be more effective for treatment of myocardial injury, and that the Ca^{2+} handling profile of iPSC-derived CMs is more cardiac-like, if the iPSCs are reprogrammed from cardiac, rather than dermal, fibroblasts.\(^5,6\) Thus, the CMs, SMCs, and ECs used in this study were generated from human, cardiac-lineage, iPSCs (hciPSCs), which had been reprogrammed from male, human, cardiac fibroblasts by transfecting the cells with lentiviruses coding for OCT4, SOX2, KLF4, and C-MYC.\(^5\) After reprogramming, the hciPSCs were engineered to constitutively express green fluorescent protein (GFP), cultured in Matrigel-coated plate with human iPSC growth medium, and regularly passaged every 6-7 days.
hciPSCs were differentiated into ECs and SMCs as described previously.\textsuperscript{7,8} Briefly, the undifferentiated cells were treated with a GSK-3β inhibitor and ascorbic acid to induce mesoderm differentiation. Five days later, cells that expressed CD34 (i.e., hciPSC-derived vascular progenitor cells [hciPSC-VPCs]) were collected via magnetic nanoparticle selection; then, the hciPSC-VCs were differentiated into ECs by culturing them on fibronectin-coated flasks with EC-developmental medium (EGM2-MV; Lonza, Basel, Switzerland) or into SMCs by culturing them on collagen IV-coated flasks with SMC-developmental medium (SmGM-2; Lonza) containing PDGF-BB and TGF-β. ECs were purified to >95% via flow-cytometry selection for both CD31 and CD144 expression and then characterized via the expression of CD31, CD144, and von Willebrand factor-8 (vWF-8). SMCs were characterized via the expression of α smooth-muscle actin (αSMA), smooth-muscle 22 alpha (SM22), and calponin.

hciPSCs were differentiated into CMs as previously reported.\textsuperscript{9} Briefly, the undifferentiated cells were expanded on a Matrigel\textsuperscript{®}-coated dish for 4 days; then, differentiation was induced on day 0 by culturing the cells with a GSK-3β inhibitor in RPMI basal medium plus B27 without insulin (B27\textsuperscript{−}). The cells were recovered 24 hours later, cultured in RPMI basal medium plus B27\textsuperscript{−} for 2 days, and then cultured in RPMI basal medium plus B27\textsuperscript{−} and a Wnt signaling inhibitor; beating cells usually appeared about 8 days after differentiation was initiated. The hciPSC-CMs were purified to >95% via metabolic selection\textsuperscript{10} and characterized via the expression of cardiac troponin I (cTnI), cardiac troponin T (cTnT), α-sarcomeric actin (αSA), Connexin 43, and ventricular myosin light chain-2 (MLC-2v).

**Creation of the human iPSC-derived cardiac muscle patches (hCMPs) and control patches**

The hCMPs (2 mm × 2 mm, 100 μm thick) were created by combining the
3D-MPE-fabricated scaffold with a 2:1:1 ratio of hciPSC-CMs, hciPSC-ECs, and hciPSC-SMCs. A total of ~50,000 cells (i.e., 12,500 cells/mm$^2$) were seeded onto the surface of the scaffold; then, the hCMPs were cultured in Dulbecco minimum essential medium (DMEM) with 10% fetal calf serum for 1 week before in vitro analyses were performed or for 1 day before in vivo transplantation. For the in-vitro experiments, control patches were created by combining the same density and ratio of hciPSC-CMs, -ECs, and -SMCs with a chemoselectively cross-linked polyethylene glycol (PEG) gel or with decellularized bovine pericardium; the control patches were also cultured in DMEM with 10% fetal calf serum for 1 week.

**Measurement of calcium transients**

hCMPs were loaded with 5 µM Fluo-4 AM (Life Technologies, USA) and incubated at 37°C and 5% CO$_2$ for 30 min; then, the intercellular AM esters were de-esterified by incubating the hCMPs with Tyrode’s salt solution at 37°C and 5% CO$_2$ for another 30 min. Intracellular calcium transients were recorded with a Zeiss Cell Observer Spinning Disk Confocal microscope equipped with a 20X (NA = 0.5) objective and an environmental chamber (37°C, 5% CO$_2$) over 30 to 120 s. Movies were analyzed with FIJI software to measure the fluorescence intensities for 2 to 8 regions of interest (F) and for 3 to 8 background regions (F$_0$) per acquisition.

**In vitro analysis of contractility**

Videos of the beating hCMPs were taken at 24 fps; then, the speed of contraction and relaxation, as well as the peak beat rate, of the hCMP was determined with motion vector analysis software developed by Huebsch, et al.$^{12}$

**Optical mapping of activation**

hCMPs were transferred to a perfusion chamber mounted on an inverted microscope.
and perfused with Hank’s balanced salt solution at 37°C. Preparations were stimulated with 2-ms rectangular pulses delivered from a bipolar electrode. Cells were stained with $V_m$-sensitive dye RH-237 (5 μM) for 5 min. To eliminate motion artifact from optical recordings, cell contractions were inhibited by supplementing staining and perfusion solutions with 10 μM of blebbistatin. Dye fluorescence was excited using an Hg/Xe arc lamp and a 560/55-nm excitation filter and measured at >650 nm using a 16x16 photodiode array at spatial resolution of 110 μm per diode, as previously described.\textsuperscript{13}

Optical signals were digitally filtered to increase the signal-to-noise ratio. Activation times were measured at the 50% level of action potential amplitude and used to construct the isochronal maps of activation spread. Conduction velocity was calculated at each recording site from local activation times and averaged across the whole map using custom-written data analysis software. Action potential durations were measured at 50% and 80% levels of repolarization ($APD_{50}$ and $APD_{80}$, respectively) as intervals between repolarization and activation times.

**Cardiac monolayer culture**

Cell suspensions containing a 2:1:1 ratio of hciPSC-CMs, -ECs, and -SMCs were seeded on a gelatin coated plate at a density of 1.2x10$^5$ cells/cm$^2$ and cultured in DMEM with 10% fetal calf serum; the culture media was changed every 2 days.

**Quantitative RT-PCR Analysis**

Total RNA was extracted by using Qiashredder and RNeasy mini kits (Qiagen, USA) as directed by the manufacturer’s instructions; then, the RNA (1-2 g per 20 L reaction) was reverse transcribed with SuperScript™ II Reverse Transcriptase (Thermo Scientific, USA) and appropriate primers (Supplemental Table 1). Quantitative PCR was performed with Maxima SYBR Green Master Mix (Thermo Scientific) on a Realplex$^2$ Real-Time
PCR system (Eppendorf, USA). Measurements were calculated via the 2−ΔCt method and normalized to glyceraldehyde phosphate dehydrogenase RNA levels.

**Murine model of myocardial infarction and hCMP administration**

All experimental procedures that involved animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota, performed in accordance with the Animal Use Guidelines of the University of Minnesota, and consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No 85-23). Twelve-week-old immunodeficient NOD-scid/γc−/− mice (Jackson Laboratory) were anesthetized with an intraperitoneal injection of sodium pentobarbital (35 mg/kg), intubated, and ventilated with a small animal respirator (Harvard Apparatus); then, a left thoracotomy was performed to expose the heart, and the left-anterior descending coronary artery was permanently ligated with an 8.0 surgical silk suture. Fifteen minutes after ligation, animals in the MI+hCMP group were treated with two hCMPs, and animals in the MI+Scaffold group were treated with two 3D-MPE–printed scaffolds that lacked any cells. Both treatments were applied to the epicardial surface over the infarcted area—the hCMP was oriented approximately parallel to the alignment of the surface myocardium and held in place by covering them with a piece of decellularized bovine pericardium that was sutured to the heart to avoid movement of hCMPs off the heart and prevent adhesions between the heart and the rib cage. Both the hCMP and the cell-free scaffold were withheld from animals in the MI group, and animals in the Sham group underwent all surgical procedures for MI induction except the ligation step and received neither of the experimental treatments. The chest was closed in layers and the animals were allowed to recover.

**Echocardiographic assessments of cardiac function**

Echocardiographic measurements were obtained via the method recommended by the
American Society of Echocardiography and performed on a Vevo770 Imaging System equipped with an RMV 707B transducer (15-45MH; VisualSonics Inc, Canada); both conventional 2-dimensional images and M-Mode images of the heart in a parasternal short axis view were acquired. Left ventricular (LV) internal diameters at end-diastole (LVIDed) and end-systole (LVIDes) were determined from 8 consecutive images, and LV ejection fractions (EF) and fractional shortening (FS) were calculated according to the following equations:

$$\text{EF} = \frac{(\text{LVIDed}^3 - \text{LVIDes}^3)}{\text{LVIDed}^3} \times 100\%; \quad \text{FS} = \frac{(\text{LVIDed} - \text{LVIDes})}{\text{LVIDed}} \times 100\%.$$

**Immunohistochemical evaluations**

The hCMPs were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, and then blocked with 5% donkey serum for 30 min. Primary antibodies (rabbit anti-cTnI [Abcam, USA], mouse anti-cTnT [Thermo Scientific, USA], mouse anti-αSMA [Sigma-Aldrich, USA], and goat anti-CD31 [Santa Cruz, USA]) were diluted in the blocking solution, and the hCMPs were incubated at 4°C; on the following day, the hCMPs were incubated with secondary antibodies for 1 h and then stained with DAPI. The alignment of cells in the hCMP channels was evaluated via multiphoton microscopy. ImageJ software was used to fit an ellipse to the outline of each cell, which was visible because of cellular autofluorescence, and the ellipse was used to determine the cell’s aspect ratio and angle relative to horizontal; then, the angle of orientation for each cell was normalized to the average longitudinal angle of the channel walls.

Murine hearts were cut into halves from the middle of the infarct. One of the halves was frozen at the optimal cutting temperature for cryo-sectioning, and the other half was stored in 10% formalin for paraffin-embedded sectioning. Embedded tissues were cut into 7-μm sections; then, the sections were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized in 0.1% Triton X-100 at 4°C for 10 min, and blocked.
with UltraV block (Thermo Scientific, USA) for 7 min. Primary antibodies (mouse anti-human specific nuclear antigen, HNA [Emdmillipore, USA], goat anti-GFP [Abcam, USA], goat anti-CD31 [Santa Cruz, USA], mouse anti-human specific CD31 [hCD31, Dako, USA], mouse anti-αSMA [Sigma-Aldrich, USA], rabbit anti-cTnI [Abcam, USA], mouse anti-cTnT [Abcam, USA], rabbit anti-Ki67 [Abcam, USA], mouse anti-αSMA [Sigma-Aldrich, USA], rabbit anti-cTnI [Abcam, USA], rabbit anti-Ki67 [Abcam, USA]) were added to the UltraV block buffer, and the sections were incubated at 4ºC. On the following day, secondary antibodies conjugated with fluorescent markers (Jackson ImmunoResearch Lab, USA) were added; then, the sections were incubated for 1 h at room temperature, stained with DAPI, washed, and examined under a confocal microscope (Zeiss 710, USA).

Engrafted hciPSC-cardiovascular cells were identified by the expression of HNA or GFP, engrafted hciPSC-CMs were identified by the expression of both GFP and cTnI, or both HNA and cTnI, engrafted hiPSC-ECs were identified by hCD31 expression, and engrafted hiPSC-SMCs were identified by the expression of both GFP and αSMA. Vascular density was evaluated by counting the number of vascular structures that were positive for either CD31 or αSMA expression; then, the images used for the CD31 and αSMA evaluations were superimposed, and arteriole density was evaluated by counting the number of structures that expressed both CD31 and αSMA. Cells and vessels were counted in 5 fields per section, 5 sections per animal.

**Engraftment rate assessment**

Engraftment rates were evaluated via quantitative PCR (qPCR) assessments of the human Y-chromosome as previous reported. Briefly, whole hearts of female mice were collected and digested overnight at 56 °C with proteinase K; then, the total DNA was isolated from the digested buffer with a QIAGEN DNA isolation kit. The number of cells in each mouse heart was determined by comparing the number of cycles required for each
sample to a standard curve calculated from the DNA of known quantities of undifferentiated hciPSCs, and the engraftment rate was calculated as the number of cells in each animal divided by the number of cells administered \(1 \times 10^5\). Analyses were performed with the SYBR Green kit (Thermo Scientific, USA) on an Eppendorf Realplex\textsuperscript{2} PCR system (Eppendorf, USA) with the following primers: sense, ATCAGCCTAGCCTGTCTT-CAGCAA; anti-sense, TTCACGACCAACAGCACAGCAATG.

To assess the engraftment rate by counting the grafted human cells as evidenced by HNA positivity, we also used histologic assessment of consecutives sections of the heart that spans the entire area covered by the engrafted patch, and counting the grafted human cells as evidenced by HNA positivity.\textsuperscript{5,15} Hearts were frozen or paraffin-embedded and transversely cut into 7-\(\mu\)m sections from base to apex. Total cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Engrafted hciPSCs-derived cardiovascular cells were identified by counting the human specific nuclear antigen (HNA)-positive cells in every 15\textsuperscript{th} serial section of the heart and then multiplying by 15 to obtain the total number of engrafted hciPSC-cardiovascular cells per heart. The engraftment rate of transplanted hciPSC-cardiovascular cells was calculated by dividing the total number of engrafted cells by the number of cells administered \(1 \times 10^5\) and multiplying by 100.

**Infarct size and thickness measurements**

Hearts were frozen or paraffin-embedded and cut into 7-\(\mu\)m sections; then, the sections were stained with an Accustain Trichrome Stains (Masson) kit (Sigma-Aldrich, USA). The infarct size was measured from Masson’s trichrome-stained sections as the percentage of the surface area of the left ventricular anterior wall occupied by scar, and infarct thickness was calculated as the ratio of the thicknesses of the fibrous region to the thickness of the septal wall. For each heart, the infarct size was averaged from three areas of the heart: just below the ligation suture, midway between the ligation suture and
the apex, and a section close to the apex.

**Apoptosis**
Sections cut from embedded hearts were TUNEL stained with an In-situ Cell Death Detection Kit (Roche Applied Science, Germany) and viewed at 40x magnification as previously reported. The number of TUNEL-positive cells and the total number of cells were counted in 5 to 6 slides per animal and 5 fields per slide.

**Statistical Analyses**
Data are presented as mean ± standard error. Statistical significance (\(P<0.05\)) between two experimental groups was determined via the Student’s two-tailed \(t\)-test, assuming unequal variance. Comparisons among more than two experimental groups were evaluated via one-way ANOVA followed by Tukey post-hoc analysis. All statistical analyses were performed with SPSS software (version 13.0, SPSS, USA).
SUPPLEMENTAL REFERENCES


8. Bao X, Lian X, Dunn KK, Shi M, Han T, Qian T, Bhute VJ, Canfield SG and Palecek SP. Chemically-defined albumin-free differentiation of human pluripotent stem cells


ONLINE FIGURE LEGENDS

Online Figure I. Immunofluorescent analysis of lineage-specific marker expression in differentiated hiPSC-derived cardiac cells. The lineage of the differentiated hciPSC-CMs was confirmed via the expression of cardiac troponin I (cTnI), cardiac troponin T (cTnT), ventricular myosin light chain 2 (MLC-2v), α-sarcomeric actin (αSA), and the gap-junction protein connexin-43 (Con-43); the lineage of the differentiated hciPSC-SMCs was confirmed via the expression of α-smooth muscle actin (αSMA), SM22, and calponin; and the lineage of the differentiated hciPSC-ECs was confirmed via the expression of CD31, CD144, and von Villebrand factor 8 (vWF-8). Nuclei were counterstained with DAPI (scale bar = 50 μm).

Online Figure II. Expression of contractile- and calcium-transient–related genes in hCMPs and in monolayers of hiPSC-derived cardiac cells. Quantitative PCR measurements of the expression of genes involved in (A) contractile activity (cTnT, cTnI, atrial myosin light chain 2 [MLC2a], MLC2v, α myosin heavy chain [αMHC], βMHC), and (B) generating calcium transients (sarcoplasmic reticulum Ca$^{2+}$-ATPase 2 α [SERCA2α], phospholamban [PLB], sodium/calcium exchanger 1 [NCX1], ryanodine receptor 2 [RYR2], calsequestrin 2 [CASQ2]) were performed in hCMPs and in monolayers composed of equivalent populations of cells on day 1 and day 7 after manufacture. Results were normalized to measurements obtained in monolayers on day 1. *P<0.05, **P<0.01; n=3-4 hCMPs or monolayers per time point with 3 replicates per measurement.

Online Figure III. hCMP transplantation reduces apoptosis and increases angiogenesis after MI. (A) Apoptotic cells were identified in sections from the border-zone of ischemia in the hearts of animals from the MI (top row), MI+Scaffold (middle row), and MI+hCMP (bottom row) groups via TUNEL staining; CMs were
visualized via immunofluorescent staining for the presence of cTnl and nuclei were counterstained with DAPI. (B) Apoptosis was quantified as the percentage of cells that were positive for TUNEL staining. (C) Sections from the border-zone of ischemia in the hearts of MI (top row), MI+Scaffold (middle row), and MI+hCMP (bottom row) animals were immunofluorescently stained for the presence of the CD31, αSMA, and cTnl, and nuclei were counterstained with DAPI; then (D) vascular density was determined by quantifying the expression of CD31, and (E) arteriole density was determined by quantifying the co-expression of CD31 and αSMA. **P<0.01, n = 5-6 in each group; scale bar = 50 μm.

**Online Figure IV. hCMP transplantation promotes cell proliferation after MI.** (A) Proliferating cells were identified in sections from the border-zone of infarct in hearts on day 28 after MI by staining for expression of the proliferation marker Ki67 (green). (B) Proliferation was quantified as the number of Ki67+ cells per high-power field (HPF). *P<0.05, **P<0.01, n=5-6 in each group. Scale bar = 100 μm.
ONLINE VIDEO LEGENDS

Online Video I. MPE-3DP to fabricate adult simulate grid layer by layer. The video shows layer-by-layer fabrication of a scaffold of dimensions 400 x 400 x 100 microns. This is the same structure as was used for the in vitro experiments. For in vivo studies, structures like the one shown here were tiled together to generate millimeter scale structures. Fabrication of each layer required approximately 10 seconds. The fluorescent contrast arises from the two-photon excitation of Rose Bengal entrapped in the matrix.

Online Video II. 360 view of 3D rendering of adult simulate grid. The video shows a 3D projection of the series of fabricated layers shown in the S1 video, with a 360 view of the fabricated structure.

Online Video III. Animation depicting the MPE-3DP process. First, a solution containing the polymer or protein of interest and associate photocrosslinking agent is added to the reservoir. The reservoir sits upon a slide on the stage of a multiphoton microscopy rig. The pulsed laser is actuated and raster scanned across the x-y plane of a given field of view. The dwell time of the laser at a given focal volume is dictated by the intensity of the digital template at a given x-y position. The longer the dwell time, the more crosslinking occurs and therefore a more stable structural feature is formed. The scan time is on the scale of microseconds for each focal volume, milliseconds for each x-y line and seconds for the entire raster (i.e., x-y plane). The animation shows a significantly slowed visual depiction of proteins of focal volumes undergoing polymerization on three different z planes. Not shown in the video is the digital template, which dictates the final form of the structure. As noted at the end of the animation, the final structure can be manipulated for use in vitro and in vivo.

Online Video IV. Spontaneous and synchronous contractile activity in a hCMP on
day 1 after fabrication (magnification: 100 x).

Online Video V. Spontaneous and synchronous contractile activity in a hCMP on day 3 after fabrication (magnification: 100 x).
## Online Table I. Primers for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Standard Gene name</th>
<th>Gene product</th>
<th>Forward</th>
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<th>Size bp</th>
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Online Figure I

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<th>hiPSC-CMs</th>
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<th>hiPSC-ECs</th>
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Online Figure II

**A**

- cTnT
- cTnI
- MLC2a
- MLC2v
- MHC
- MHC
- Actinin2

**B**

- SERCA2
- PLB
- NCX1
- RYR2

Legend:
- □ Monolayer, day 1
- ■ hCMP, day 1
- □ Monolayer, day 7
- □ hCMP, day 7

* Statistical significance compared to Monolayer, day 1
** Statistical significance compared to Monolayer, day 7
Online Figure III

A

B

Percentage (TUNEL+ cells/Total cells)

C

D

Vascular density (mm²)

E

Arteriole density (mm²)

* * * * *
Online Figure IV

A

Ki67 DAPI

MI

MI+Scaffold

MI+hCMP

B

<table>
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<th>Treatment</th>
<th>Ki67 cells/HPF</th>
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<tr>
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<tr>
<td>MI+hCMP</td>
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* p < 0.05
** p < 0.01