Supplemental Information

Cardiac Stem Cell Patch Integrated with Microengineered Blood Vessels Promotes Cardiomyocyte Proliferation and Neovascularization After Acute Myocardial Infarction

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Teng Su, Ke Huang, and Michael A. Daniele contributed equally to this work.

1. Materials and Methods

1.1 Cell isolation and culture

Human CSCs were derived from donor human hearts. All procedures were approved by the institutional review board and written informed consent was obtained from all patients. Myocardial tissues were minced into small pieces ($1 - 2$ mm^3), washed with phosphate buffered saline (PBS), and then digested with collagenase solution (Sigma-Aldrich). The tissue fragments were cultured as cardiac explants on a plate coated with 0.5 mg·mL^{-1} of human fibronectin (Corning) in CSC growth medium comprising Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% fetal bovine serum (Corning), 0.5% gentamycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), and 1% L-glutamine (Invitrogen) at 37 °C in 5% CO_2. After a period ranging from 1 to 2 weeks, a layer of stromal-like cells was generated from adherent cardiac explants over which small, round, phase-bright cells migrated. These explant-derived cells were harvested and then seeded at a density of 2×10^4 cells·mL^{-1} in ultralow attachment flasks (Corning) for cardiosphere formation. After about 1 week, loosely adherent cardiospheres formed from the explant-derived cells were harvested and plated in fibronectin-coated flasks (Corning) for expansion as cardiosphere-derived CSCs. The CSCs were used at passage 2 – 4. Primary HUVECs were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were expanded in the HUVEC growth media consisting of vascular cell basal medium (ATCC PCS-100-030) supplemented with endothelial cell growth kit (ATCC PCS-100-041) and used at passage 4 – 6.

1.2 Fabrication of BMVs

To fabricate the BMVs with HUVECs lining the luminal surface, a microfluidic device consisting of a central microchannel having two shaping regions was utilized. Each shaping region is patterned with a set of four chevron-shaped grooves in the channel walls. The HUVECs comprising the lumen were suspended at a density of 3×10^7 cells·mL^{-1} in a 1 wt % gelatin (from porcine skin, Sigma-Aldrich) dissolved in HUVEC growth media to form the core solution. For the cladding solution, gelatin

methacrylamide (GelMA) was mixed with 4-arm poly (ethylene glycol)-tetra-norbornene (PEGN; 10 kDa, Sigma-Aldrich) and poly (ethylene glycol)-tetra-thiol (PEGT; 10 kDa, Sigma-Aldrich) at a weight ratio of 2:1:1 in PBS containing the photoinitiator 2-hydroxy-1-(4-(2-hydroxyethoxy) phenyl)-2-methyl-1-propanone (Irgacure 2959; Sigma-Aldrich). The total polymer concentration of GelMA-PEGN-PEGT mixture was 6 wt %. Fibronectin (Corning) was added at 50 $\mu g \cdot mL^{-1}$ to supplement the GelMA-PEG mixture for further supporting cellular attachment. The outer sheath stream was made of 6 wt % PEG suspended in PBS. Hydrodynamic focusing in microfluidic channels was used to direct these three solutions into coaxial flow patterns. The flow rates of the core, shell and sheath streams were 15, 30, and 90 $\mu L \cdot min^{-1}$, respectively. The BMVs were generated by UV irradiation at 365 nm at a density of approximately 10 $mW \cdot cm^{-2}$ and then maintained in HUVEC growth media at 37 °C in 5% CO_2. The media were changed every other day.

1.3 Cell viability and proliferation in the BMVs

The HUVEC viability at various time points (day 1, 3, 14, and 28) after culture in the BMVs was measured using the Live/Dead cell staining assay (Invitrogen) according to the manufacturer's instruction. The live cells were labeled green with calcein-AM and dead cells were labeled red with ethidium homodimer (EthD-1). The BMVs were gently rinsed, followed by incubation with 2 μM calcein-AM and 4 μM EthD-1 in PBS for 30 min at 37 °C. After incubation, the stained samples were washed thrice and immediately analyzed using fluorescent microscopy (Olympus IX81). The live and dead cells in the fluorescent images of BMVs were quantified using NIH ImageJ software. At each time point, 3 BMV samples were selected for imaging. The numbers of live cells (green) and dead cells (red) from 5 microscopic fields were averaged and reported. The cell viability was calculated as (number of live cells / total number of the live and dead cells) × 100%.

To evaluate the cell proliferation in BMVs, quantification of cellular DNA was performed. The HUVEC-laden BMVs produced in 1 min (containing approximately 4.5×10^5 cells as estimated from the cell concentration and flow rate of core stream) by using the microfabrication method described

above were collected for the assay. The BMVs were maintained in HUVEC growth media at 37 °C in 5% CO_2. The media were changed every other day. After 1, 3, 14, and 28 days of culture, the cell-laden microvessels were lyophilized and digested by incubating with 6 $U \cdot mL^{-1}$ of collagenase (Sigma-Aldrich) in TNE buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, 2 mM sodium azide, pH 7.4) at 37 °C for 2 h. The digests were subjected to a freeze-thaw cycle and spun down at 16000 × g for 5 min. 100 μL of 10-fold diluted sample supernatant was then incubated with 100 μL of Hoechst 33258 working solution (2 $\mu g \cdot mL^{-1}$) in TNE buffer. The fluorescence measurement was performed using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific) with excitation and emission at 360 nm and 460 nm, respectively. The number of cells in the BMVs was determined by comparing the fluorescence intensity of the sample solution with a calibration of fluorescence intensity for known cell numbers under the same assay conditions (Figure S13). The cell density, namely the number of HUVECs per mm^3, in the BMVs was determined by dividing the cell number at various time points by the volume of the BMVs (i.e., the volume of cladding fluid dispensed per min).

1.4 Immunofluorescence staining of cultured BMVs

After 7 or 14 days of *in vitro* culture, BMVs were harvested and gently rinsed in PBS (pH 7.4) prior to fixation with 4% paraformaldehyde for 30 min at room temperature. After that, the samples were permeabilized and blocked with Protein Block Solution (DAKO) containing 0.1% saponin (Sigma-Aldrich), and then incubated with the following primary antibodies overnight at 4 °C: mouse anti-human CD31 (1:500, ab24590, Abcam), rabbit anti-human Ki67 (1:200, ab15580, Abcam), and goat anti-human ZO-1 tight junction protein (1:500, ab190085, Abcam). Then the samples were incubated with goat anti-mouse IgG-FITC conjugate (1:600, ab6785, Abcam), goat anti-rabbit IgG-Cy3 conjugate (1:600, ab6939, Abcam), and donkey anti-goat IgG-Cy5 conjugate (1:600, ab6566, Abcam) for 3 h at room temperature. DAPI (Invitrogen) was used for counterstaining the cell nuclei. Images were taken by a Zeiss LSM 710 confocal microscope (Carl Zeiss, Germany) and analyzed using NIH ImageJ software. At each time point, 5 BMV samples were selected for imaging. The numbers of Ki67 positive

or ZO-1 positive cells from 5 high-power microscopic fields (HPF, × 40 objective) were averaged and reported.

1.5 Fabrication of cardiac patches

To fabricate the BMV-CSC patch, the BMVs after being cultured for 17 days were arranged biaxially on the bottom surface of one well of a 12-well plate (Figure S14). Fibrin gel kit (Tisseel; Baxter) was used to encapsulate both the BMVs and the human CSCs. Briefly, the human CSCs were suspended in basal IMDM and placed in the fibrinogen-containing chamber of the dual-barrel syringe provided in the kit. The thrombin component was loaded into the other syringe chamber. The final composition of the fibrin gel consisted of 37 $mg \cdot mL^{-1}$ of fibrinogen, 8.0×10^6 human CSCs per mL, 217 $U \cdot mL^{-1}$ of thrombin, and 1270 $U \cdot mL^{-1}$ of aprotinin. Approximately 385 μL of fibrin gel solution was ejected through the tip of the dual-barrel syringe into one well of the 12-well plate. The fibrin gel was incubated at 37 °C in 5% CO_2 for 15 min to allow complete polymerization before the addition of a 1:1 combination of HUVEC growth medium and CSC growth medium. The as-prepared fibrin gel disk was cut into square pieces (5 mm × 5 mm × 1 mm) with each piece containing 6 biaxially aligned BMV segments to produce the BMV-CSC patches.

For the fabrication of HUVEC-CSC patch, we determined the number of HUVECs embedded in the BMV-CSC patch using the following method. The cell density in the BMV after 17 days of culture (D_{17}) was determined by using the DNA quantification method described above. The cross-sectional area of BMV (S_c) was calculated from the outer and inner diameters of microvessel wall. As each BMV-CSC patch contains 6 pieces of 5-mm-long BMV segments, the number of HUVECs embedded in the BMV-CSC patch was determined using the formula $D_{17} \times S_c \times 30$. Therefore, approximately 385 μL of fibrin gel solution containing 37 $mg \cdot mL^{-1}$ of fibrinogen, 3.96×10^6 HUVECs per mL, 8.0×10^6 human CSCs per mL, 217 $U \cdot mL^{-1}$ of thrombin, 1270 $U \cdot mL^{-1}$ of aprotinin, and IMDM was ejected into one well of a 12-well plate. The as-prepared gel disk containing randomly distributed HUVECs and human CSCs was cut into square pieces (5 mm × 5 mm × 1 mm) to give the HUVEC-CSC patches.

The CSC patch was fabricated in the same manner but without the addition of HUVECs. The native fibrin gel patch was designated as empty patch. All the patches were maintained at 37 °C in 5% CO_2 and fed every day for 8 days prior to transplantation.

1.6 Morphological analysis

The morphology of the microvessels and cardiac patches was observed by using a JEOL JSM-7600 field emission scanning electron microscope (JEOL, USA) equipped with a Gatan Alto-2500 cryogenic system (Gatan, USA) at an accelerating voltage of 5 kV. The specimen was placed on the sample holder, plunge-frozen in liquid nitrogen and transferred to the preparation chamber where it was fractured at -170 °C. The fractured sample was sublimed at -90 °C to eliminate any presence of condensed ice and then sputter-coated with a thin layer of Au/Pd prior to observation.

1.7 Rheological measurement

The viscoelastic behavior of fibrin gel patches was measured using a StressTech rheometer (ATS RheoSystems, USA) with parallel plate geometry (diameter: 20 mm) at 20 °C. The fibrin gel patches embedded with biaxially aligned BMVs were prepared in a similar manner to the BMV-CSC patches in a 12-well plate, except that CSCs were not included. The fibrin gel patch without BMVs served as control. The patches were cut into disk-shaped specimens of 20 mm in diameter and 1 mm in height. Frequency sweep experiments measured the storage modulus (G') and loss modulus (G'') as a function of angular frequency (ω, $1 - 100$ rad$\cdot$s^{-1}). Dynamic time sweep experiments measured the storage and loss moduli as a function of time at a constant frequency of 6.28 rad$\cdot$s^{-1} (1 Hz). All measurements were carried out at applied stress values that were determined to be within the linear viscoelastic regime by an initial stress sweep. The data were averaged from three independent measurements.

2. Figures

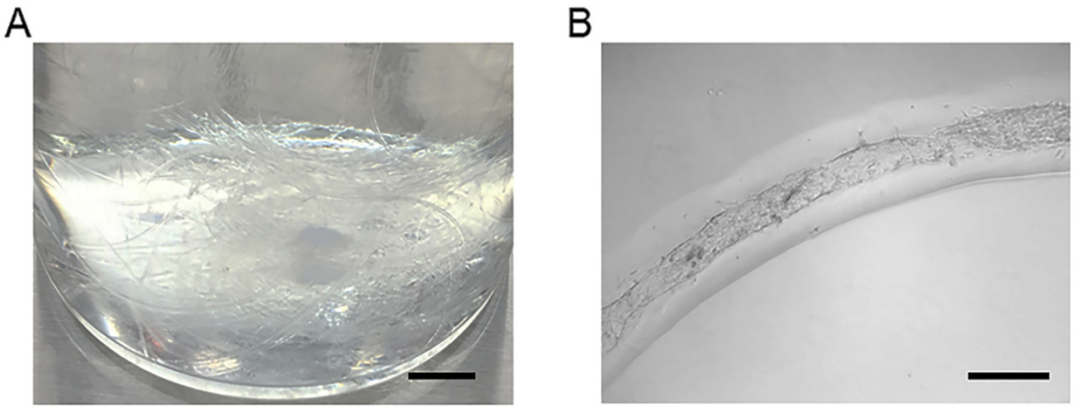

Figure S1. Macroscopic images showing a representative BMV with a length greater than one meter (A) after fabrication and an enlarged BMV with a confluent monolayer of HUVECs lining the luminal surface (B) after 14 days of culture**.** Scale bars, 5 mm (a); 500 μm (b).

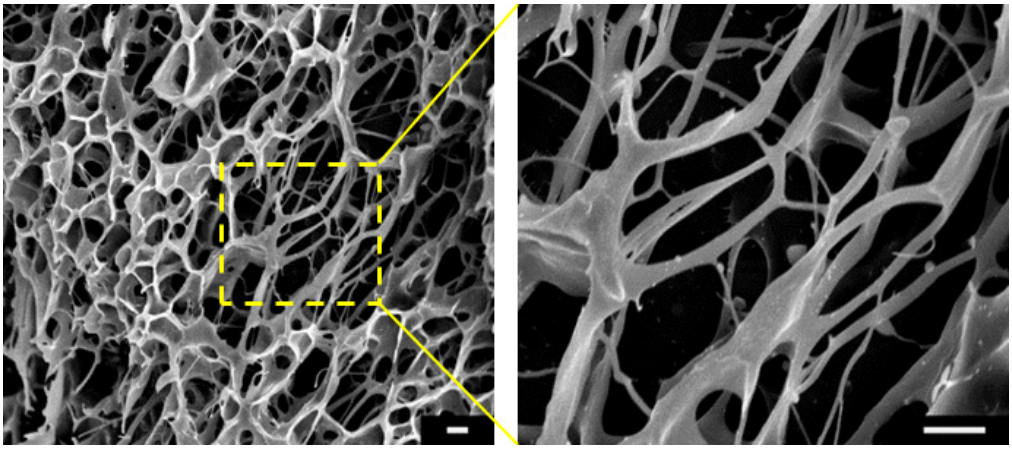

Figure S2. SEM images showing the microstructure of the cardiac patch without BMVs and human CSCs. Scale bars, 1 μm.

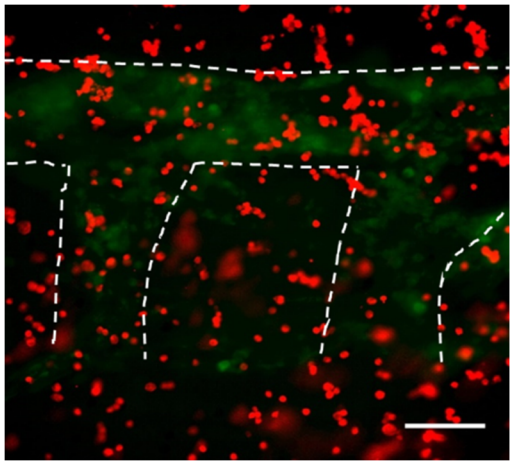

Figure S3. Fluorescent image of the BMV-CSC patch after 22 days of *in vitro* culture. HUVECs were pre-labeled with DiO (green) and human CSCs were pre-labeled with DiI (red) before fabrication. Scale bar, 250 μm.

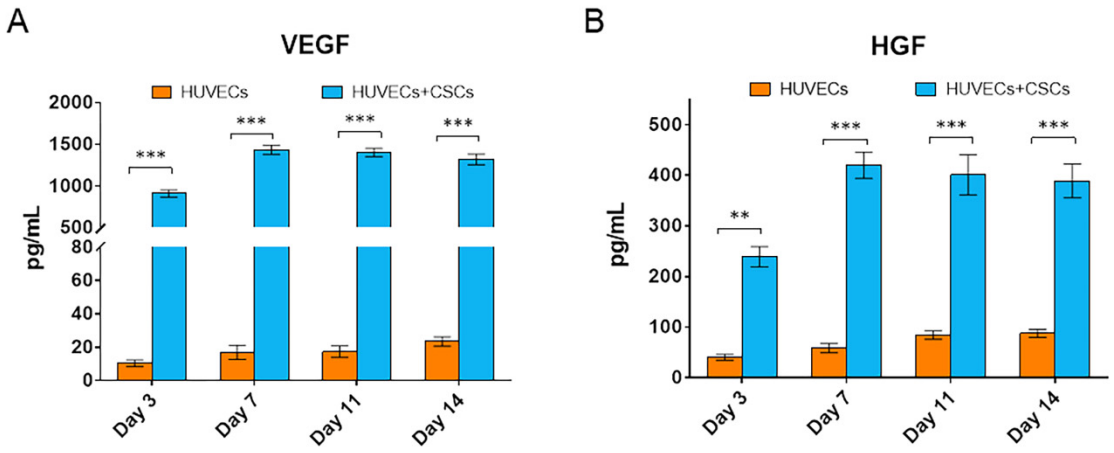

Figure S4. The release of VEGF (A) and HGF (B) in the conditioned media of solitary HUVEC culture versus the co-culture of HUVECs and CSCs with equivalent endothelial cell number on standard tissue culture plate. n = 3 for each group at each time point. All data are mean ± s.d. ** indicates $p < 0.01$, *** indicates $p < 0.001$. Comparisons between any two groups were performed using two-tailed unpaired Student's *t*-test.

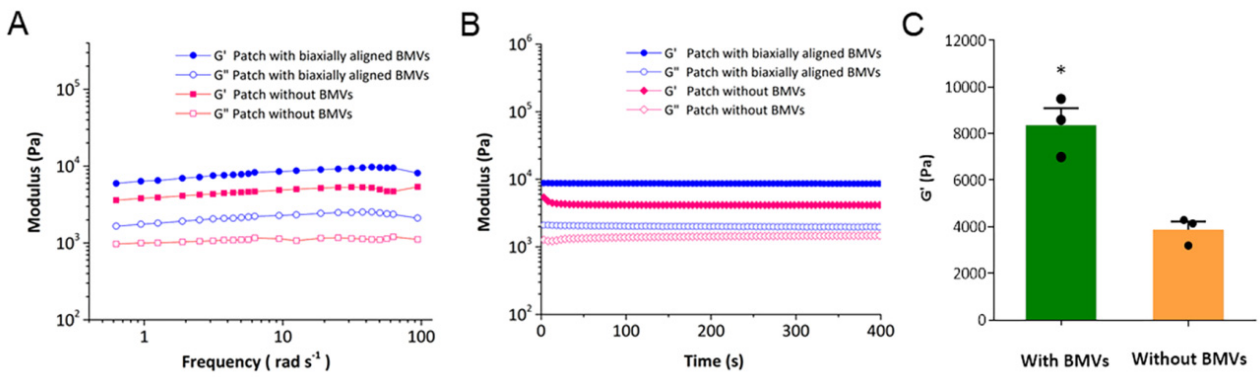

Figure S5. (A, B) Viscoelastic behavior of the cardiac patches with or without biaxially aligned BMVs during dynamic frequency sweep (A) and dynamic time sweep (B). (C) Comparison of the storage modulus of different patches. n = 3 independent experiments. All data are mean ± s.d. * indicates $p < 0.05$. Comparisons between any two groups were performed using two-tailed unpaired Student's *t*-test.

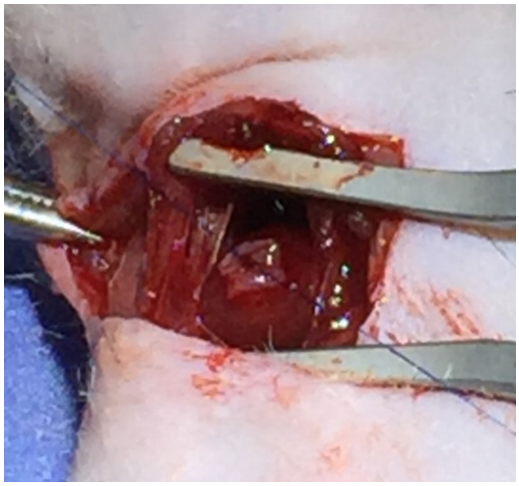

Figure S6. Representative macroscopic image showing a BMV-CSC patch after being sutured on the epicardial surface of the left ventricle immediately after MI induction.

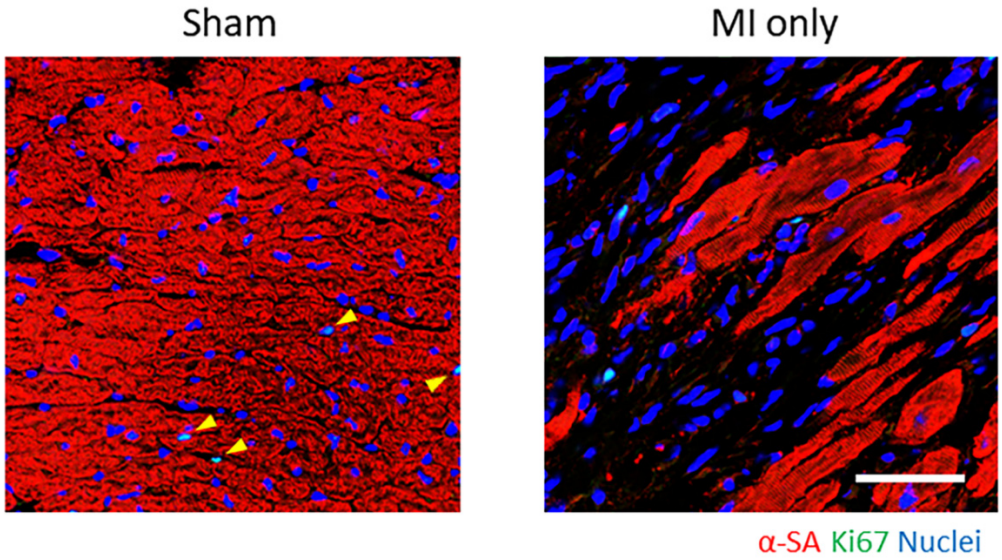

Figure S7. Representative images showing Ki67-positive cardiomyocytes (yellow arrowheads) in the sham and MI-operated hearts at week 4. Scale bar, 50 μm.

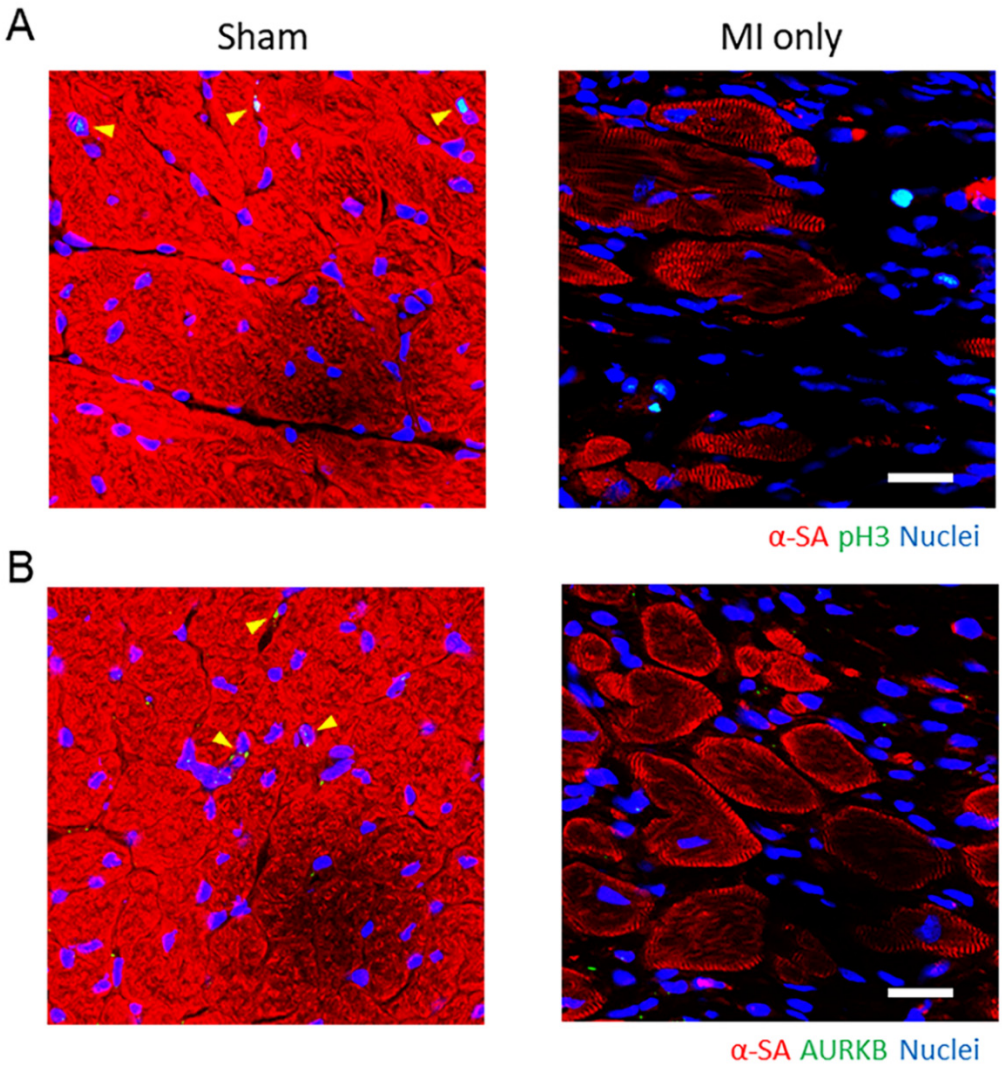

Figure S8. Representative images showing pH3- (A) or AURKB- (B) positive cardiomyocytes (yellow arrowheads) in the sham and MI-operated hearts at week 4. Scale bars, 20 μm.

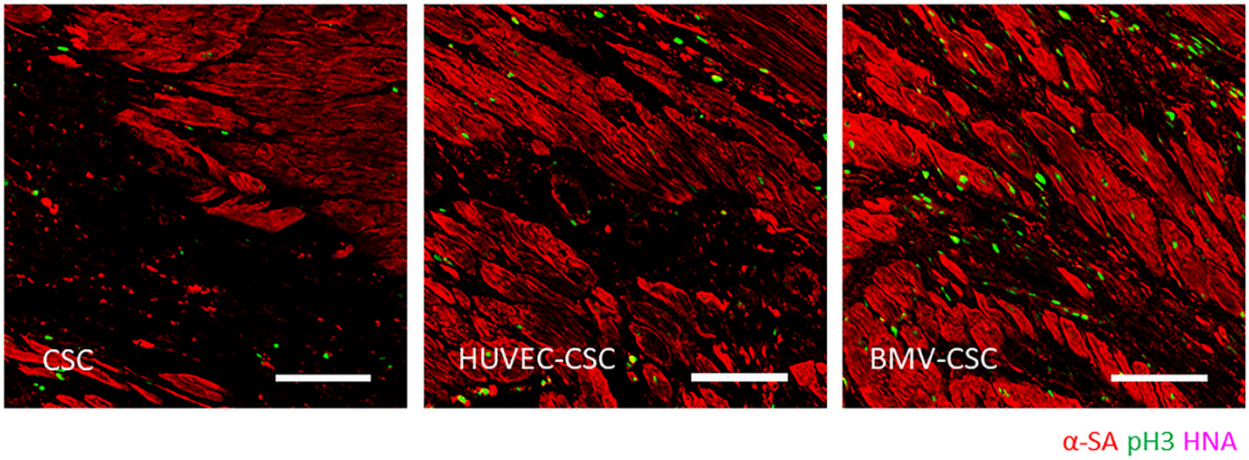

Figure S9. Representative images showing that the pH3-positive proliferating cardiomyocytes in CSC patch-, HUVEC-CSC patch-, and BMV-CSC patch-treated hearts at week 4 were not expressing human nuclear antigen (HNA). Red: α-sarcomeric actinin (ab9465, Abcam), staining cardiomyocytes; green: pH3 (ab5176, Abcam), indicating the cells that are in late G2/mitosis phase; magenta: HNA (ab191181, Abcam). The proliferating cardiomyocytes did not express HNA, indicating that these cells were of endogenous origin. Scale bars, 100 μm.

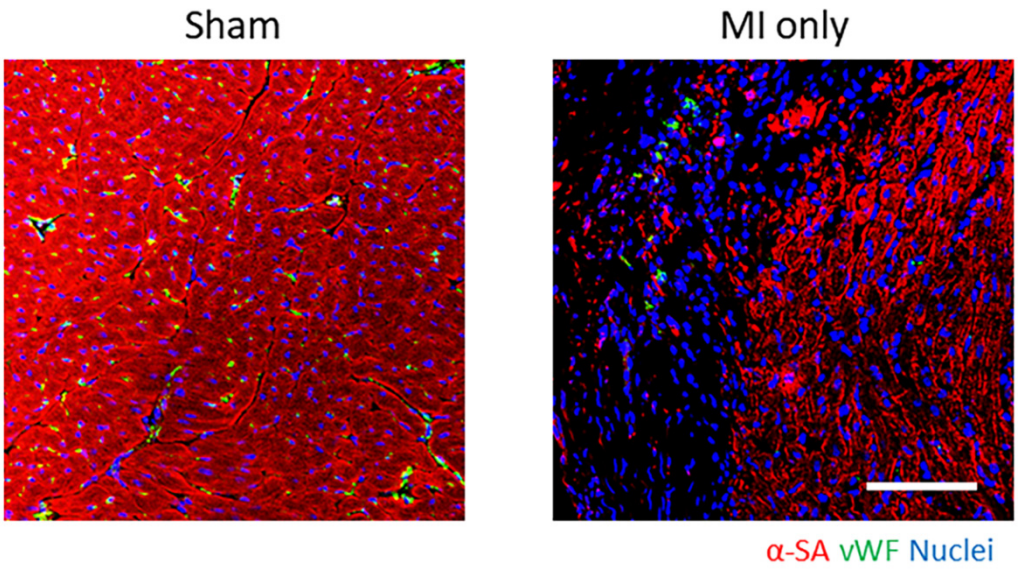

Figure S10. Representative images showing vWF-positive vasculatures (green) in the sham and MI-operated hearts at week 4. Scale bar, 100 μm.

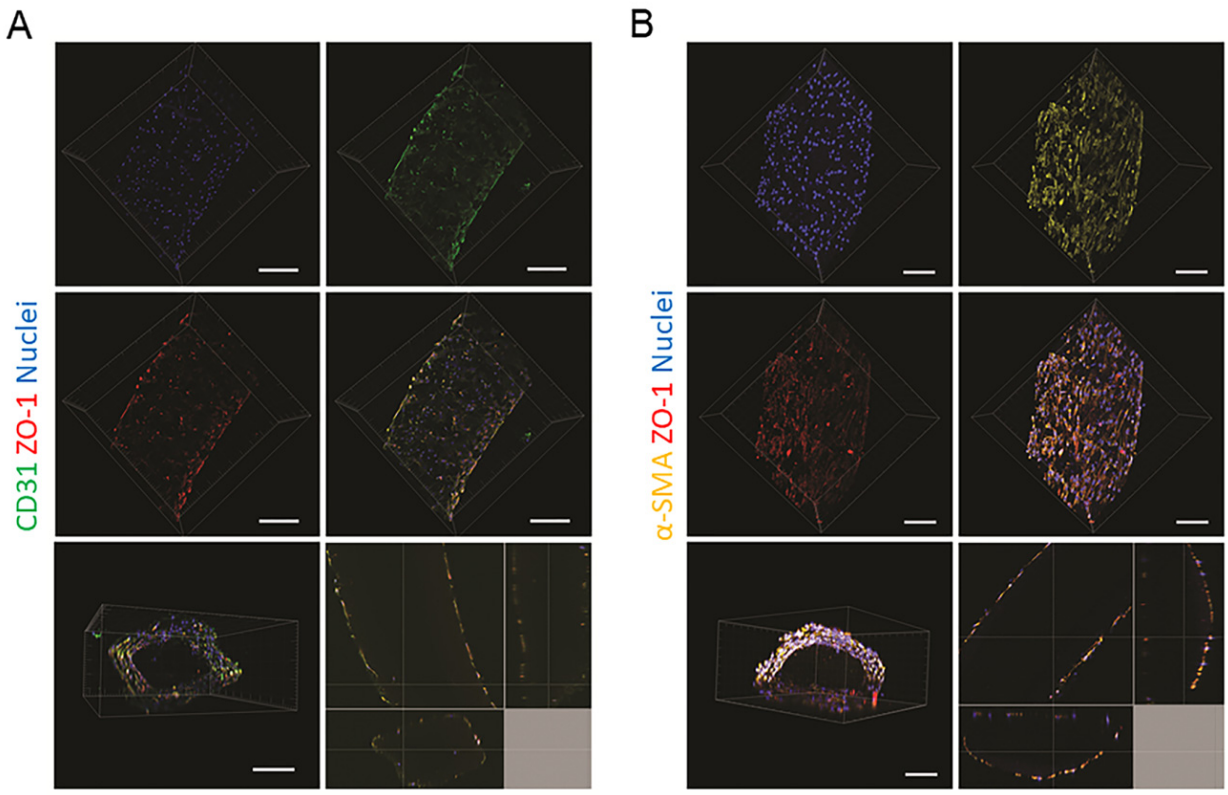

Figure S11. Hydrodynamic focusing microfluidic fabrication supports the generation of microvessels that incorporate different cell types. A subpopulation of HUVECs (A) or human aortic smooth muscle cells (B) can form tubule-like morphology in the wall of BMVs. Scale bars, 250 μm.

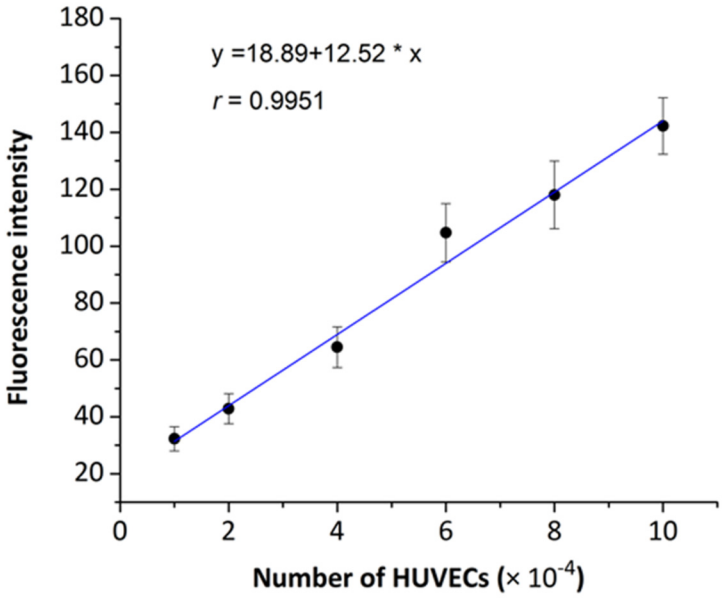

Figure S12. The standard curve constructed for determining the cell proliferation in BMVs based on DNA quantification.

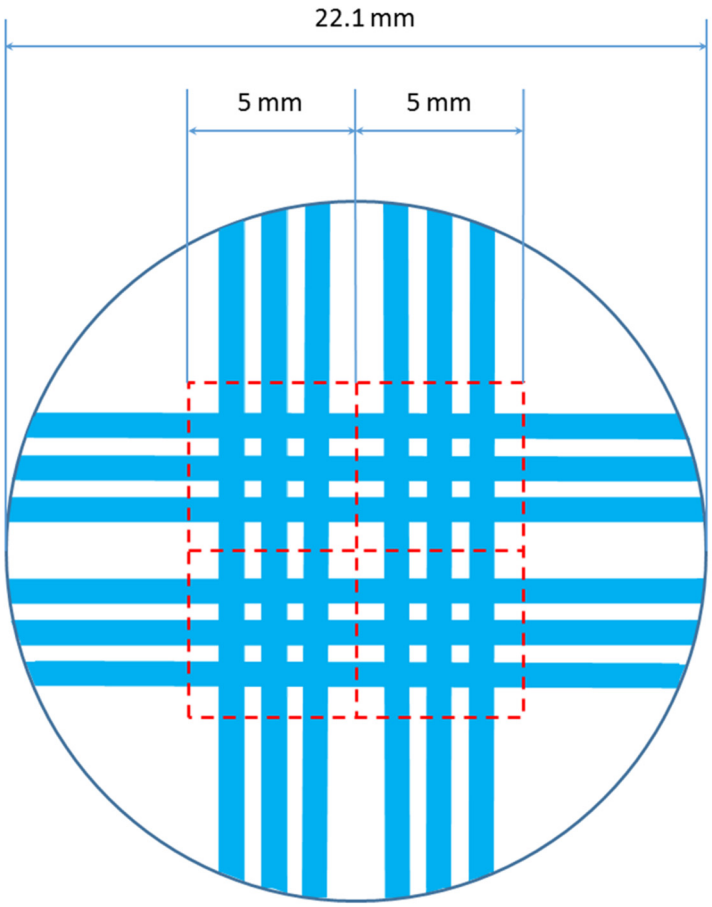

Figure S13. Schematic description of fabricating the BMV-CSC patches in one well of a 12-well cell culture plate. The BMVs highlighted as blue stripes were biaxially arranged in the well before casting the fibrin gel containing human CSCs. The red dashed lines denote the cutting positions to produce the BMV-CSC patches.