Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts

Meredith A. Redd1,2,3, Nicole Zeinstra1,2,3, Wan Qin1, Wei Wei1, Amy Martinson2,3,4, Yuliang Wang3,5, Ruikang K. Wang1, Charles E. Murry1,2,3,4,6 & Ying Zheng1,2,3

Vascularization and efficient perfusion are long-standing challenges in cardiac tissue engineering. Here we report engineered perfusable microvascular constructs, wherein human embryonic stem cell-derived endothelial cells (hESC-ECs) are seeded both into patterned microchannels and the surrounding collagen matrix. In vitro, the hESC-ECs lining the luminal walls readily sprout and anastomose with de novo-formed endothelial tubes in the matrix under flow. When implanted on infarcted rat hearts, the perfusable microvessel grafts integrate with coronary vasculature to a greater degree than non-perfusable self-assembled constructs at 5 days post-implantation. Optical microangiography imaging reveal that perfusable grafts have 6-fold greater vascular density, 2.5-fold higher vascular velocities and >20-fold higher volumetric perfusion rates. Implantation of perfusable grafts containing additional hESC-derived cardiomyocytes show higher cardiomyocyte and vascular density. Thus, pre-patterned vascular networks enhance vascular remodeling and accelerate coronary perfusion, potentially supporting cardiac tissues after implantation. These findings should facilitate the next generation of cardiac tissue engineering design.
Engineered tissues have emerged as promising approaches to repair damaged organs as well as useful platforms for drug testing and disease modeling. However, insufficient vascularization is a major challenge in engineering complex tissues such as the heart. Heart failure is the leading cause of death worldwide, and no available treatment options outside of whole heart transplantation address the problem of cellular deficiency. Despite this burgeoning clinical need, the therapeutic application of engineered cardiac tissues has not been achieved, partially due to the lack of comprehensive tissue perfusion in vitro and effective integration with host vessels in vivo.

Prior efforts to vascularize tissue grafts have mostly relied on self-assembly of endothelial cells (ECs) to form connected tubes within cardiac constructs. Although the presence of these vessels improves cardiomyocyte maturation and tissue function, the formed network architecture does not provide efficient perfusion, preventing large-scale construct fabrication and culture. When implanted, these grafts partially integrate with host vasculature but do not establish effective perfusion in a timely fashion. To combat this problem, efforts have been made toward fabricating perfusable vasculature within cardiac tissue constructs in our laboratory and in others. Little is known, however, about how these vascular networks will connect with host vessels once implanted and whether physiological systemic perfusion in the grafts can be established.

An engineered tissue also requires appropriate cell sources, which are not only important to promote tissue function but also critical for clinical translation. In particular, the field of vascularization has mostly relied on human umbilical vein endothelial cells (HUVECs), a commonly used endothelial source with known function and availability but poor survival and immunogenic issues in vivo. Our laboratory has demonstrated that we can use human pluripotent stem cells to derive ECs (human embryonic stem cell-derived endothelial cells (hESC-ECs)) and cardiomyocytes from mesodermal precursors. Importantly, these hESC-ECs exhibit increased angiogenic behavior in flow-derived microphysiological constructs and are vasogenic when embedded in bulk hydrogel matrix. These properties indicate that hESC-ECs could be an ideal cell source for engineering constructs with high vascular density.

As vascular engineering strategies continue to advance, it is critical to develop better systems to measure perfusion dynamics and achieve more efficient graft–host integration. Standard approaches to assess the graft integration rely on the presence or absence of red blood cells or perfused lectins in histological sections. It has not been possible to directly measure flow and perfusion in the graft and new coronary vasculature. We recently demonstrated an application of optical coherence tomography (OCT)-based optical microangiography (OMAG) to obtain high-resolution coronary angiograms on ex vivo Langendorff-perfused and fixed rat hearts. This imaging technique allows for simultaneous image acquisition of high-resolution structural information as well as velocimetry data of the coronary vasculature in both graft and host.

In this study, we combine advanced tissue engineering, stem cell biology, and ex vivo intact heart imaging techniques to study the vascular anastomosis and host integration in the infarcted heart. We demonstrate vascular remodeling and anastomosis in vitro between pre-patterned, perfusable vascular networks and self-assembled (SA) vessels in the bulk matrix, both with hESC-EC cell sources. We show that remodeled constructs with vascular anastomosis have upregulated genes associated with vascular and tissue development. Importantly, these pre-patterned, perfusable constructs improved vascular host integration, which likely supported graft cardiomyocyte remodeling when implanted on an infarcted heart compared to SA controls. Our work demonstrates that pre-perfused, patterned vessels provide important cues for rapid anastomosis and host integration and sheds light on engineering translational cardiac patches for heart regeneration.

Results

Engineering human stem cell-derived microvasculature. To engineer human stem cell-derived microvessels (µVs) in vitro, we first generated ECs, previously called endocardial-like ECs, from a dual reporter RUES2 human stem cell line (mTmG-2a-puro RUES2) using our recently established differentiation protocol. These stem cells stably express either TdTomato red fluorescent protein or (following Cre-mediated recombination) green fluorescent protein (GFP), allowing us to evaluate vascular remodeling between two separately seeded vascular compartments (Supplementary Figure 1A, B). Both mTm- and GFP-expressing hESC differentiated into endocardial progenitors by differentiation day 5 with >70% populations expressing CD34, a surface marker expressed by both hematopoietic and endothelial populations (Supplementary Figure 1C, D). After an additional week in endothelial culture conditions, endocardial populations arose with >98% purity by day 14 as shown by the expression of endothelial junction protein, CD31 (Supplementary Figure 1E, F). These cells widely express the endocardial transcription factor, NFATC1, throughout the culture (Supplementary Figure 2). We previously demonstrated that these differentiated ECs undergo tubulogenesis when embedded in soft collagen gels and extensive angiogenesis when lined in a three-dimensional (3D) engineered µV platform.

Here we further developed constructs with enhanced vascularity by combining the lithographically defined network and SA de novo small tubes of hESC-ECs and microfluidic channel networks were fabricated in collagen gel matrices with and without bulk-seeded GFP-hESC-ECs. The patterned network was then seeded with mTm-hESC-ECs to form the endocardium-lined lumen (Fig. 1a). After culturing under gravity-driven flow for 4 or 7 days, mTm-hESC-ECs in the lumen formed patent µV that retained the original network geometry (Supplementary Figure 3A) and sprouted extensively into the bulk matrix (Fig. 1b, c). GFP-hESC-ECs in the matrix underwent tubulogenesis to form SA lumens in the surrounding collagen (Supplementary Figure 3B). Throughout culture, the ECs retained robust expression of endothelial markers such as vascular endothelial cadherin, VE-cadherin, CD31, and von Willebrand factor (VWF) (Fig. 1c and Supplementary Figure 3C, D). The newly formed SA tubes integrated with angiogenic sprouts from the µV network and formed numerous anastomotic connections (Supplementary Figure 2E and Supplementary Movie 1). Anastomosis was observed between GFP+ de novo tubes and mTm+ endothelium through direct connection at the patterned µV or to the smaller angiogenic sprouts (Fig. 1d, Supplementary Figure 3E). Likewise, many GFP-hESC-ECs did not form de novo tubes, but rather incorporated directly into the vessel wall of the both the sprouts and the pre-patterned µV (Fig. 1d).

To evaluate the effect of de novo lumen formation and anastomosis on the structure and function of the patterned endothelium, we quantified angiogenesis in µVs generated with (µV+SA) and without (µV only) bulk-seeded GFP-hESC-ECs and cultured for 4 or 7 days. While hESC-ECs remodeled extensively and sprouted into the collagen matrix in both groups, the overall number of mTm+ sprouts per vessel surface area was not significantly different between the two groups or from day 4 to day 7 in culture (Fig. 1e). The sprout length and diameter, however, increased over time between 4 and 7 days of culture in µV only constructs, with an overall increasing trend in both sprout length and diameter in the µV+SA constructs over time.
and compared to \( \mu V \) only (Fig. 1e). This suggests that the interstitial de novo tubes help establish and stabilize endothelial sprouts from the patterned \( \mu V \)s. 3D rendering of confocal z-stacks further confirmed the extensive integration and anastomosis between GFP+ lumens near the patterned, perfused network and the microvascular spouts that penetrated deep into the interstitial collagen (Fig. 1f).

We next analyzed de novo lumen density and average lumen size with respect to the distance from the \( \mu V \) wall in \( \mu V + \)SA constructs. GFP+ lumen density was significantly decreased with increased distance from the vessel wall after 7 days in culture with a similar trend after just 4 days (Supplementary Figure 4A-D). The density of GFP+ cells near the vessel wall was comparable from day 4 to day 7 suggesting that the decline in EC density at the larger distances (>600 \( \mu m \)) was likely due to lumen regression or cell death rather than migration (Supplementary Figure 4D). Ethidium homodimer-1 staining showed trends toward decreasing cell viability with increased distance from the vessel wall and was lower at day 7 compared to day 4 (Supplementary Figure 4E).

The average size of GFP+ lumens near the vessel wall (within 300 \( \mu m \)) was significantly larger than lumens located >600 \( \mu m \) distance away after 4 and 7 days of culture (Supplementary Figure 4F). These findings suggest that perfusion promotes better de novo lumen formation, which in turn leads to better remodeling and anastomosis.

### In vitro perfusion of \( \mu V \)s and angiogenic sprouts

We examined the flow and perfusion characteristics of engineered \( \mu V \)s and endothelial sprouts in \( \mu V \) only and \( \mu V + \)SA constructs. Fluorescent beads, perfused from the vessel inlets, were observed to immediately fill the endothelial-lined, patterned microchannels before moving into the endothelial sprouts (Supplementary Movie 2 and Fig. 2a). By comparing bead perfusion images with immunofluorescent confocal images, we confirmed that sites of anastomosis between the de novo lumens and the patterned \( \mu V \)s were patent and perfusable (Fig. 2a, b). The total perfused area of the constructs increased with time in both \( \mu V + \)SA and \( \mu V \) only groups but was not significantly different between the two groups (Supplementary Figure 5A-C). This suggests that substantial vascular remodeling occurred over time, but the addition of perfusable anastomotic connections in \( \mu V + \)SA constructs was not yet sufficient to influence the global perfusion dynamics of the constructs. There was no significant difference in flow velocity in the pre-patterned channel (diameter >100 \( \mu m \)) in both vessel conditions (Fig. 2c). In sprouts with diameters <50 \( \mu m \), the average bead velocity was significantly higher in \( \mu V + \)SA (87.9 ± 5.6 \( \mu m/s \), mean ± standard error) compared to \( \mu V \) only constructs (42.6 ± 8.4 \( \mu m/s \)) after 7 days of culture (Fig. 2d), whereas differences in day 4 constructs were not significant. An upward shift in sprout velocity was observed across all diameters between 10 and 50 \( \mu m \) in day 7 \( \mu V + \)SA constructs (Fig. 2e). Flow simulation
of idealized sprouted vessel networks showed that the addition of sprouts led to higher flow in the vessel networks when the same pressure drop is applied between an inlet and an outlet, indicating a lower flow resistance (Supplementary Figure 5D). Together, these data suggest that localized vascular remodeling and anastomotic events decrease the vascular resistance, allowing more efficient perfusion through endothelial sprouts.

**Stem cell-derived µVs are non-thrombogenic**. Next, we investigated the interaction between blood and hESC-EC-derived engineered µVs. Citrate-stabilized, ABO-matched whole blood with labeled platelets was perfused from the inlet and into the µV lumens. Most blood flowed freely and continuously through the patterned µV and exited from the outlet throughout 20–30 min of perfusion (Supplementary Movie 3 and Fig. 3a). Some blood cells entered the endothelial sprouts (Supplementary Movie 3) and eventually stopped, presumably due to their dead-end architecture. In regions where sprouts connected two branches of the patterned vessel, individual red blood cells could be seen passing through the newly formed anastomosis bridges (Supplementary Movie 4). Few red blood cells clumped or adhered to the vessel throughout the blood perfusion, and these could be completely washed out of the main vessel without obstructing flow (Supplementary Figure 6A). Small amounts of platelets, labeled with antibodies to CD41a, aka platelet-specific glycoprotein 11b, adhered to the vessel wall of engineered µVs but without formation of large aggregates (Supplementary Movies 5 and 6). Platelet adhesion remained at a low level throughout the blood perfusion over 20 min (Supplementary Figure 6B), although there was somewhat greater adhesion in the hESC-EC constructs than in quiescent HUVEC-seeded µVs28. Subsequent immuno-fluorescent analysis further confirmed low platelet adherence on the walls of hESC-EC endothelium, and platelets (CD41a) that were adhered were primarily localized to endothelial junctions (CD31) (Fig. 3b, c, Supplementary Figure 6C, D). When activated with phorbol myristate acetate (PMA) or interleukin (IL)-1β, the hESC-EC µVs showed increased platelet adhesion after 20 min of blood perfusion (Fig. 3b, c, Supplementary Figure 6E, F). These findings demonstrated that hESC-EC-formed µVs are non-thrombogenic and can convert to a thrombogenic state as a physiological response to stimuli.

**Unique µV gene expression of vascular development**. To better understand the difference among SA only, µV only, and µV+SA constructs, we collected RNA for transcript profiling using RNAseq analysis for the three groups after 3 days of culture in vitro. The µVs in the µV only and µV+SA constructs were made from a large grid pattern (13 × 13) with lumen diameter of 125 μm to maximize the vascular surface and RNA yield. Although we predicted that the µV+SA constructs would have an intermediate profile between the µV only and the SA only constructs, this was not the case. Strikingly, >5000 genes were significantly different (fold change >1.5 and false discovery rate (FDR) <0.05) in µV+SA constructs compared to SA constructs, whereas approximately 500 genes were significant when comparing µV vs. SA constructs. We performed principal component analysis (PCA), which showed that PC1 separated the µV+SA constructs from the other two, whereas in PC2 the µV+SA constructs were intermediate between the µV only or the SA only constructs (Fig. 4a). The top genes that differentiated the µV+SA
Fig. 3 Citrated whole-blood perfusion in engineered microvessels (µVs). a Brightfield stitched large image of red blood cell-filled pattern and sprouts with magnified view (inset, white dotted boundary) for human embryonic stem cell-derived endothelial cell (hESC-EC)-seeded µV only constructs after 4 days of culture. Scale bar, 200 µm. b Maximum intensity projection of confocal z-stack of constructs with adhered platelets after 30-min perfusion and subsequent phosphate-buffered saline (PBS) washes for untreated hESC-EC-seeded constructs (top) and phorbol myristate acetate (PMA)-treated hESC-EC-seeded constructs (bottom) stained for CD31 (red) and CD41a (green). Scale bar, 200 µm. c Quantification of platelet adhesion on the vessel wall for constructs seeded with human umbilical vein endothelial cells (HUVECs) in control conditions (C-HUVEC), hESC-ECs in control conditions (C-hESC-EC), and hESC-ECs treated with PMA (PMA-hESC-EC) or interleukin (IL)-1β (IL-1β)-hESC-EC. Data are expressed as a percentage of the vessel wall surface area. N = 2, 2, 2, and 3 biologically independent samples for C-HUVEC, C-hESC-EC, PMA, and IL-1β, respectively. Representative images for a, b from two biologically independent samples of C-hESC-ECs and two biologically independent samples of PMA-hESC-EC, with similar results. Error bars, mean ± SEM.

Graft vascular perfusion by OMAG. To determine the extent of vascular integration between the rat myocardium and the implanted vascular grafts, we performed ex vivo real-time imaging of graft perfusion prior to histological processing of the hearts. OMAG scans of the coronary vasculature were collected during Langendorff perfusion. The coronary flow was driven by an aortic perfusion pressure of 90 mm Hg, which is within the range of normal diastolic aortic pressures for both healthy rat and human hearts. Two OMAG imaging protocols, OMAG and OMAG-V (see Methods), were used in this study in order to acquire high-resolution images of vascular structure and quantitative blood velocities within the grafts. In normal healthy regions of the heart, OMAG and OMAG-V images revealed dense vasculature with hierarchical branching structure and high flow rates in large arteries when compared to smaller vessels (Fig. 5a). µV only constructs showed minimal vascular flow at 5 days after implantation (Supplementary Figure 9A), and therefore, these were not included in the velocity measurements. Considerable vascular flow was observed in the pre-patterned, perfusable vascular grafts (µV+SA) with higher velocities in larger diameter vessels, whereas the SA grafts had few visible perfused vessels (Fig. 5b, c). Since OMAG data acquisition and processing uses sequential frames to distinguish moving from stationary particles, a single 3D scan gives rise to both structural information and vascular flow. To distinguish between host and graft, OMAG flow data were overlaid with structural information extracted from the same image scan (Supplementary Movies 7 and 8), and 3D images were generated.
number of genes scans (Supplementary Figure 9B) 35. The average measured correlation between velocity and signal intensity in OMAG-V of individual vessels in the grafts were assessed by using the linear SA and both groups was comparable at 63.5 ± 9.2% and 64.5 ± 5.1% for area). Vessel area density in neighboring non-infarcted regions of 5.6% of tissue area) compared to SA grafts (5.3 ± 1.7% of tissue area). This perfusion rate is >20-fold greater than that of the SA myocardium and significantly higher than that of SA grafts (0.29 ± 0.10 mm/s) (Fig. 5f). The perfusion rate for the field of view was 24.5 ± 8.8 µL/min in µV+SA grafts, corresponding to a volumetric perfusion rate of approximately 12.3 ± 4.4 mL/min/mL tissue. This perfusion rate is >20-fold greater than that of the SA grafts which had a perfusion rate of 0.9 ± 0.8 µL/min (0.5 ± 0.4 mL/min/mL tissue) (Fig. 5g). Despite this improvement, perfusion rates in both grafts were significantly less than rates in non-infarcted healthy regions of the same hearts (117.9 ± 32.6 µL/min (Fig. 5d). This overlay reveals drastic improvement in vascular perfusion of µV+SA grafts compared to SA grafts. Quantification of the overall vessel area density in OMAG images confirmed that µV+SA grafts had significantly more perfused vessels (33.8 ± 5.6% of tissue area) compared to SA grafts (5.3 ± 1.7% of tissue area). Vessel area density in neighboring non-infarcted regions of both groups was comparable at 63.5 ± 9.2% and 64.5 ± 5.1% for SA and µV+SA grafts, respectively (Fig. 5e). Perfusion dynamics of individual vessels in the grafts were assessed by using the linear correlation between velocity and signal intensity in OMAG-V scans (Supplementary Figure 9B)35. The average measured velocity (vessel diameter between 20 µm and 40 µm) in µV+SA grafts (0.72 ± 0.09 mm/s) was similar to that of the non-infarcted myocardium and significantly higher than that of SA grafts (0.29 ± 0.10 mm/s) (Fig. 5f). The perfusion rate for the field of view was 24.5 ± 8.8 µL/min in µV+SA grafts, corresponding to a volumetric perfusion rate of approximately 12.3 ± 4.4 mL/min/mL tissue. This perfusion rate is >20-fold greater than that of the SA grafts which had a perfusion rate of 0.9 ± 0.8 µL/min (0.5 ± 0.4 mL/min/mL tissue) (Fig. 5g). Despite this improvement, perfusion rates in both grafts were significantly less than rates in non-infarcted healthy regions of the same hearts (117.9 ± 32.6 µL/min
Human μVs survived implantation and were perfused. To identify and distinguish between perfused human vessels vs. infiltrating host vessels in the grafts, we perfused lectins through the ex vivo rat hearts prior to histological processing. Two types of lectins were retrograde perfused: fluorescein *Griffonia simplicifolia* Lectin I (GSL I) and rhodamine *Ulex europaeus* Agglutinin I (UEA I) to label rat and human endothelium, respectively. A non-specific lectin antibody was then used to recognize all perfused vessels (of both rat and human origin), while an anti-rhodamine antibody was used to specifically detect UEA I+ perfused human vessels within the graft. Antibodies against TdTomato (anti-DsRed) identified all human ECs (Fig. 6a, b, Supplementary Figure 10A). The presence of human vessels was confirmed with additional staining for human endothelial junction protein, hCD31 (Supplementary Figure 10B-D). Quantification of the density and size of hESC-EC vessels in the grafts showed no difference between μV+SA and SA groups (Fig. 6c). The density of total perfused vessels (lectin+), however, was greater in μV+SA constructs (373 ± 94 vessels/mm²) compared to SA grafts (149 ± 55 vessels/mm²), along with an increasing trend in the average vessel size (Fig. 6c). The number of perfused vessels in grafts is still less than the host infarct region (healthy region of SA), 113.9 ± 16.9 μL/min (healthy region of μV +SA)). These results demonstrate that engineered vascular constructs integrate functionally with the host vasculature and that pre-patterned, perfusable vasculature improves graft perfusion dynamics.
(Supplementary Figure 10E). To specifically identify human vessels that integrated with host circulation, we quantified UEA1+ vessels, which was higher in μV+SA grafts (29.6 ± 17.0 vessels/mm²) compared to SA grafts (4.2 ± 1.6 vessels/mm²). The fact that the perfused human vessels were 10% of the total perfused vessels indicates that most of graft perfusion was due to host vascular ingrowth (Fig. 6d). Smooth muscle encoating within graft human vessels was observed, but in rare events in vascular grafts, and did not appear to be different between groups (Supplementary Figure 10B–D). These findings confirm histologically that our composite perfusible vascular grafts are better perfused and integrated with host coronary circulation than SA non-perfusable grafts and that the implanted human ECs contributed to this effect.

**Pre-patterned cardiac grafts survived implantation and were perfused.** To determine the benefit of increased early perfusion of engineered μV grafts, we implanted both perfusible (μV+SA) and non-perfusable (SA) cardiac constructs containing additional hESC-derived cardiomyocytes (hESC-CMs) onto infarcted athymic Sprague-Dawley rat hearts using the previously described procedure for vascular graft implantations. The cardiac constructs contained hESC-CMs and human bone marrow-derived stromal cells HS-27a in addition to hESC-ECs at a ratio of 4:1:1, respectively, in bulk matrix. hESC-ECs were also seeded and cultured under perfusion within the pre-patterned lumens as described earlier. All hESC-CM populations used for cardiac constructs had cardiomyocyte purity >70% as indicated by the expression of cardiac marker cTnT (Supplementary Figure 11A,

**Fig. 6** Detection of human endothelial cells and perfused vessels in grafts 5 days post-implantation. **a, b** Immunofluorescent-stained paraffin sections of self-assembled (SA) and μV+SA grafts: **a** human embryonic stem cell-derived endothelial cells (hESC-ECs) (DsRed+, red) and perfused rat and human endothelium (Lectin+, green). White arrows in merged images denote double positive cells. Scale bar, 100 µm. **b** hESC-ECs (DsRed+, red) and perfused human endothelium (UEA I+, white). Yellow arrows in merged images denote double positive cells. Scale bar, 100 µm. **c** Quantification of vessel density (number of vessels per mm²) and average vessel size for hESC-EC vessels (DsRed+, red circles) and all perfused vessels (Lectin+, blue circles) in the grafts. N = 6 biologically independent animals for both SA and μV+SA grafts, p = 0.045 for lectin+ vessels for SA and μV+SA, p = 0.018 for DsRed+ and lectin+ for μV+SA, p > 0.05 for all others (two-tailed t test). **d** Quantification of vessel density and average vessel size of perfused human vessels (UEA I+, grey circles) in the grafts. N = 6 biologically independent animals for both SA and μV+SA grafts, with one animal with μV+SA graft excluded from vessel size calculation due to lack of UEA I+ vessels. p = 0.023 for vessel size for SA and μV+SA, p > 0.05 for vessel density (two-tailed t test). Representative images for **a, b** from six biologically independent animals containing SA grafts and six biologically independent animals containing μV+SA grafts, with similar results. Hoechst-stained nuclei, blue. Data were collected from at least three confocal images of randomly selected regions per sample and analyzed by a custom lumen identifying code for vessel density and size. Error bars, mean ± SEM. *p < 0.05 determined using two-tailed t test. UEA Ulex europaeus Agglutinin I
B). After 4 days of culture, spontaneous beating was detected in both µV+SA and SA cardiac constructs (Supplementary Movies 9 and 10). At 5 days post-implantation, hearts were excised, and histological analysis was performed. Both groups had similar infarct sizes (Supplementary Figure 11C-D). To assess hESC-CM graft formation, we stained for human-specific β-myosin heavy chain (β-MHC) (Fig. 7a, b). The density of β-MHC+ cells was significantly greater in the µV+SA cardiac grafts (194 ± 31 cells/mm²) than the SA grafts (85 ± 12 cells/mm²). The β-MHC+ graft size, normalized by the left ventricle size, was measured to be greater, however, not significantly, in µV+SA grafts than SA grafts (0.16 ± 0.05% and 0.11 ± 0.02% respectively; p = NS, two-tailed t test) (Fig. 7c). This suggests that perfusable µVs enhanced hESC-CM remodeling, which could potentially improve cardiac engraftment and function long term. The total perfused vessel density was also significantly greater in µV+SA cardiac grafts (320 ± 53 vessels/mm²) than in SA cardiac grafts (179 ± 24 vessels/mm²) (Fig. 7d). Additionally, the average lumen size was greater, though not significantly, in µV+SA grafts (75 ± 32 μm) than in SA grafts (40 ± 8 μm) (Fig. 7d). We next sought to determine whether cells within the grafts were viable and performed a terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assay to mark apoptotic cells (Fig. 7e). Both µV+SA and SA grafts had low levels of apoptotic cells (1.1 ± 0.5% and 1.9 ± 0.5%, respectively; p = NS, two-tailed t test) (Fig. 7f). To specifically determine hESC-CM viability, we performed a TUNEL assay with an α-actinin co-stain to label cardiomyocytes. We were unable to identify any TUNEL+/α-actinin+ cells, indicating that remaining cardiomyocytes had high viability and possibly passed the peak cell death at 5 days post-implantation (Supplementary Figure 11E). These findings demonstrate the beneficial role of a perfusable, patterned vascular network in cardiac graft remodeling, which could lead to enhanced engraftment and function long term.

Discussion

Vascularization and rapid host integration remain as critical challenges for engineered cardiac tissues and for their successful translation to the clinic for heart regeneration. While considerable progress has been made to generate pre-vascularized tissues, efficient perfusion has not been achieved in vitro or in vivo when implanted on injured myocardium. In this study, we successfully engineered highly vascularized constructs from stem cell-derived ECs by incorporating SA capillary tubes around a patterned and perfusable microvascular network. We demonstrated that pre-patterned, perfusable µVs underwent extensive remodeling and anastomosis with de novo capillary tubes in vitro, and the resulting perfusable vascular construct systemically integrated with host vasculature after implantation better than non-patterned, non-perfusable constructs.

Our study demonstrated the successful use of hESC-ECs and potentially patient-derived induced pluripotent stem cells as the vascular cell source in engineered tissue constructs. The use of a patient-specific autologous cell source would bypass the immunogenic risks associated with clinical transplantation. In addition, the use of ECs at the same developmental stage as the cardiomyocytes may provide important signaling to promote cardiomyocyte maturation and tissue function. Our work took a step beyond EC differentiation from hESCs and further built 3D µVs that allow for robust culture and phenotype maintenance under flow. We demonstrated and utilized their angiogenic, tubulogenic, and nonthrombogenic properties toward engineering highly vascularized constructs for tissue engineering applications.

Our studies uncover that perfusion facilitates anastomosis and vascular remodeling in vitro. We showed that anastomosis occurs between de novo capillary tubes and pre-patterned vascular conduits and that bulk-seeded ECs directly incorporate into the microvascular endothelium, leading to increased flow velocity and decreased vascular resistance. This phenomenon appears similar to the incorporation of endothelial progenitor cells into active sites of angiogenesis in animal models of ischemia. Transcriptional analysis further revealed unique gene clusters toward upregulated signaling in hypoxia, glycolysis, and vascular development in anastomosed constructs. These studies demonstrate the formation of a highly remodeled perfusable vascular network with numerous anastomotic connections, and more importantly, these vessels are capable of carrying blood without blockage.

Our implantation studies quantitatively demonstrate that perfusable vascularized grafts integrated significantly better than non-perfusable SA vascular grafts. We exploited OCT-based imaging technology in the intact heart to assess the perfusion dynamics in grafts under physiological pressure, which had not been achieved previously. The live heart poses a major challenge for assessment of coronary flow due to large motion artifacts. Our use of diastolically arrested, fixed hearts allowed for precise control of the applied pressure at physiological level for flow measurement while eliminating the motion artifacts associated with the beating heart. While this is admittedly different from a living, beating heart, it does present a snapshot of the heart’s microcirculation at the peak phase of coronary perfusion. This, in combination with intralipid perfusion to mimic blood flow and generate angiograms, made it possible to obtain the 3D vascular structure, flow velocity, and perfusion rate of the vasculature down to the capillary scale in tissue grafts implanted on infarcted hearts. More importantly, our approach allowed for unambiguous distinction between the host and graft with respect to their tissue structure and perfusion dynamics. Our patterned vascular grafts at 5 days post-implantation demonstrated comparable perfusion velocities (0.72 mm/s in 20–30 μm sized vessels) to those in the non-infarcted myocardium as well to previously reported values in similar sized vessels and arterioles in living tissues.

The ability to quantify the perfusion dynamics of implanted vascular grafts ex vivo could provide a more precise readout for comparing different vascularization strategies to achieve better transport efficiency, which would benefit future development of vascular engineering techniques. One limitation to this approach is the inability to distinguish human vessels from rat vessels during the real-time OMAG data acquisition. In this study, we relied on subsequent histological detection to confirm the role of implanted human cells, which limits the information that can be gathered on the extent the cells within the patterned network contributed to the increased perfusion dynamics. A system that has dual imaging capabilities with both OCT and fluorescent-based imaging could potentially be used to overcome this challenge.

Despite a normal range of velocities displayed in vessels within patterned vascular grafts, these newly integrated vessels do not yet structurally resemble proper coronary structure. The graft vessels are more sparsely distributed with less organized structures compared to the dense, aligned vasculature displayed by their healthy counterparts. Surprisingly, the overall structure of perfused graft vessels did not retain the original geometry of the network pattern, but the pre-patterning did enhance vascular density and perfusion. Our histological assessment suggested that, although the implanted human ECs did contribute to the newly connected and perfused vessels in the graft, most perfused vessels originated from the host. This suggests that the pre-patternning
**Fig. 7** Perfusion cardiac constructs in infarcted rat heart model 5 days post-implantation. 

**a** Immunofluorescent-stained paraffin sections of self-assembled (SA; left) and μV+SA (right) cardiac grafts: Whole-graft section containing human embryonic stem cell-derived cardiomyocytes (hESC-CMs) (β-MHC, red). Gray dotted line outlines graft tissue. Scale bar, 1 mm. 

**b** High magnification images of boxed region in a (yellow box) with hESC-CMs (β-MHC, red, bottom right) and perfused vessels (Lectin+, green, bottom left). Merged, top. Scale bar, 100 μm. 

**c** Quantification of cardiomyocyte density (number of β-MHC+ cells per mm²) and β-MHC+ graft size (% LV area). *N* = 7 and 6 biologically independent animals for SA and μV+SA, with one animal with μV+SA graft excluded from graft size calculation due to partial graft removal during tissue processing. *p* = 0.015 for density, *p* > 0.05 for graft size (two-tailed *t* test). 

**d** Quantification of vessel density (number of vessels per mm²) and average vessel size for all perfused vessels (Lectin+) in the cardiac grafts. *N* = 7 and 6 biologically independent animals for SA and μV+SA, respectively. *p* = 0.046 for vessel density, *p* > 0.05 for vessel size (two-tailed *t* test). 

**e** Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling (TUNEL) assay for apoptotic cells (TUNEL, green) on paraffin sections of SA (left) and μV+SA (right) cardiac grafts. Scale bar, 100 μm. 

**f** Quantification of apoptotic cells (TUNEL+) as a percentage of all cells (human and host) in the graft. *N* = 7 and 6 biologically independent animals for SA and μV+SA, respectively. *p* > 0.05 (two-tailed *t* test). Representative images for **a**, **b**, **e** from six biologically independent animals containing SA grafts and five biologically independent animals containing μV+SA grafts, with similar results. Hoechst-stained nuclei, blue. Data were collected from at least two confocal images of randomly selected regions per sample. Images analyzed by a custom lumen identifying code for vessel density and size. Error bars, mean ± SEM. *p* < 0.05 determined using two-tailed *t* test. β-MHC beta-myosin heavy chain. 
determines host vessel infiltration patterns and leads to better vascular structure and function.

Indeed, several studies have shown that prior vascular patterning in grafts promotes better overall integration and structure and function.

In order to investigate the effects of pre-vascularization on graft integration, we used a grid-like pattern to fabricate the microfluidic vessels in a perfused matrix, which helps guide coronary in vivo integration. The pre-vascularized grafts were then implanted into the host vasculature, where they continued to grow and integrate with the host vessels.

To further investigate the role of pre-vascularization, we used a perfusion system to simulate blood flow through the grafts. This system allows for controlled delivery of growth factors and other biological signals to the grafts, which can influence their growth and integration.

In conclusion, our studies demonstrate the importance of pre-vascularization in improving graft integration and function. These findings have significant implications for the development of improved vascular prostheses and tissue engineering applications.
One-hour incubation with the following secondary antibodies, conjugated primary antibodies, and nuclear counterstains was performed at room temperature: sheep anti-rabbit Alexa Fluor 488 (Invitrogen A11055, 1:100), donkey anti-rabbit Alexa Fluor 594 (Invitrogen A21207, 1:100), and Hoechst 33342 (Sigma, 1:250). 3D z-stack images were acquired on a Nikon A1R confocal microscope with all image post-processing and quantification done by the ImageJ software. GFP-HESC-EC density and lumin size were quantified in µV+SA constructs by applying a threshold to maximum intensity projections (MIPs) of the GFP channel followed by ImageJ particle analysis (particles <20 μm² in size excluded to account for background) within the specified distance of the µVs. CD31-stained µVs (0–300 μm), [300–600 μm], and [600–900 μm]. Endothelial sprouts were quantified with either DsRed-stained µVs (µV+SA) or CD31-stained µVs (µV only) to determine sprouting from the patterned µV itself. GFP-HESC-ECs also express CD31. Sprouts were manually counted and their lengths and diameters measured using 3D orthogonal views and z-stack images in ImageJ. The number of sprouts was normalized to the surface area of the endothelium. After all confocal images were collected, vessels were perfused with 50 μL of 1.0 μm diameter red fluorescent beads (Thermo Fisher F13083, 1 x 10^10 beads/mL, 580/605) diluted 1:30 in PBS. Brightfield and fluorescent time series were collected with no delay at x10 magnification on a Nikon high-resolution wide-field microscope. Bead velocity was quantified by using ImageJ manual particle tracking in both sprouts and the patterned microchannels (> 100 μm). Perfusion area was quantified by measuring the total area of perfused beads in large images that were manually stitched together with the ImageJ software from smaller ×10. For each vessel, the stitched image encompassed a field of view twice the area of the original pattern boundary.

**Whole-blood perfusion and analysis of platelet accumulation.** Fresh blood (in 0.129 M sodium citrate) was obtained through Puget Sound Blood Center under IRB approval. Platelets in the platelet-rich plasma (PRP) were labeled with 0.129 M sodium citrate) was obtained through Puget Sound Blood Center under IRB approval. Platelets in the platelet-rich plasma (PRP) were labeled with fluorescein isothiocyanate-conjugated CD41a antibody (BD 555466, final concentration of 2.5 μg/mL) for 30 min at room temperature. The labeled PRP were reconstituted with red blood cells and buffy coat at the original ratio to produce reconstituted whole blood, which was perfused through the live µVs that had first been washed with PBS. For activated µVs, 50 nM PMA or 1 μg/mL IL-1β were perfused through the vessel lumen for 30 min before PBS wash. The blood flow was set for 15–30 min so that the wall shear stresses are between 10 and 30 dynes/cm². All blood perfusion experiments were done in mTh-hESC-ECs seeded µVs that were perfused for 15–20 min and then left in a no flow state for 10 min. Flow was collected at x10 magnification on a Nikon high-resolution wide-field microscope to visualize red blood cell movement and platelet accumulation of the vessel walls. At the end of blood perfusion, the vessels were washed with PBS for three times to remove the excessive blood-cell suspensions and fixed in situ using 3.7% formaldehyde. The vessels were washed with PBS three times and immunohistochemically stained for CD31 and VWF48 and imaged on a Nikon A1R confocal microscope to obtain image stacks along z direction. Using the ImageJ software, the projection of image stacks were made and platelet adhesion was quantified by measuring the area of CD41+ fluorescent signal and normalizing to vessel wall surface area.

**RNA isolation and RNAseq data analysis.** Total RNA from three types of constructs was purified using the RNAasy Mini Kit (Qiagen) and residual DNA was removed by on-column DNase digestion. RNA quality was assessed with the Agilent RNA 6000 Nano Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples with RNA integrity number >8 were kept for further analysis. RNA sequencing was performed on poly-A-enriched samples using Illumina TrueSeq. RNAseq samples were aligned to hg38 using TopHat v2.1.04. Gene-level read counts were generated with htsset-count v0.6.1p49 using the intersection-strict overlapping mode. Genes with >1 reads per million in at least 2 samples were kept for further analysis. The GLM method in edgeR v3.18.1 was used for differential expression analysis. The log2 fold change >1.5 and FDR < 0.01 were considered differentially expressed. Hallmarks gene set51 was used for pathway enrichment analysis. Hypergeometric test was used to test the significance of overlap between upregulated/downregulated genes and a pathway.

**Tissue harvest and retrograde perfusion fixation.** At their experimental endpoint, the rats were euthanized with a chemical overdose of pentobarbital/physiological saline solution (20% pentobarbital intraperitoneal injection). Once the animal was deeply anesthetized but while the heart was still beating, the chest was opened and 50 U Heparin was intravenously infused via the inferior vena cava and allowed to circulate for 1–2 min to prevent thrombosis in the coronary vessels. Intravenous infusion with supersaturated potassium chloride (KCl) was then used to arrest the heart in diastole followed immediately with excision of the heart. The aorta was cannulated followed by retrograde perfusion with a vasodilator buffer (PBS containing 4 mg/L Papaverin and 1 g/L adenosine) followed by 4% formaldehyde perfusion for 10 min. Perfusion pressure was maintained at ~100 mm Hg. After perfusion fixation, the hearts were transfused to fresh fixative overnight at 4°C. After overnight fixation, the whole perfused hearts were transferred to PBS buffer and then transferred on ice for OMAG imaging.

**OMAG assessment of vascular flow in grafts and normal healthy heart.** To image vascular flow, the hearts were retrograde perfused with 10% Intralipid (Sigma)38 and imaged with OCT-based technology52. During imaging, the hearts were placed on a custom-built imaging platform with rotational control and secured cannula to prevent tissue movement55. Perfusion pressure was maintained at ~100 mm Hg. After the OCT acquisition, two parameter protocols that covered the same 1.5 x 1.5 mm² field of view were used to acquire data for both OMAG and OMAG-V. Both protocols shared the same system setup with conventional fiber-based spectral domain OCT, which has been described in detail in a previous publication56. Briefly, this section will describe the specific procedures that determine the best protocols that allow the function of blood vessels ex vivo. The light source was a super-luminescent diode (LS2000b, Thorlabs Inc.) with a spectral bandwidth of 110 nm at 3 dB and operated at a center...
wavelength of 1340 nm. A ×10 telecentric objective lens was adopted to focus the beam spot onto the heart sample with an incident light power of ~1.9 mW. The axial resolution was 5 μm and the lateral resolution was ~7 μm in air. The maximal imaging speed of the system reached 92,000 A-lines per second, and the corresponding system sensitivity was ~100 dB in focus position at ~600 μm below the zero delay line. The system ranging depth was measured to be 3.5 mm in air.

For OMAG, raster beam scanning was performed to capture 250 A-lines within each B-frame (2D cross-section), 4000 B-frames for each C-scan (3D volume), and 16 frame repetitions at each of the 250 A-line locations to achieve high vasculature contrast. A single 3D dataset was obtained in 15 s by using a frame rate of 280 frames/s. The 3D volumetric vasculature were used to calculate vessel density as a percentage of the imaging field of view.

For OMAG-V, 200 B-frames were captured in each C-scan. Within each frame, 10,000 A-lines were obtained that were comprised of 50 repeated A-lines at 200 positions. By utilizing a defined system speed of 20,000 A-lines per second, the 3D velocimetry data were acquired in 2.5 min. An inter-frame ultrahigh-sensitive OMAG algorithm35 was utilized to extract 3D vasculatures from heart tissue image structures. By MIP of the volumetric vasculature, morphological information of the microvascular network can be visualized from the top view. In order to quantitatively analyze the flow velocity in capillaries, Eigen decomposition statistical analysis was applied to the repeated A-lines35,36. In brief, the repeated A-line ensembles were first stacked into a covariance matrix and then this matrix was represented in terms of its eigenvalues and eigenvectors through diagonal factorization. Therefore, the frequency of flowing intralipid particles can be calculated by the first lag-one autocorrelation of the eigenvectors35,36. Finally, the velocity of flow in vessels with diameters between 20 and 40 μm was assessed according to its linear correlation with the measured frequency35. Graft perfusion rates for the imaging field of view were calculated by multiplying the velocities with the perfused area and normalizing to the perfusion rates of the corresponding healthy region. All analysis of animal experiments was performed by a blinded observer without knowledge of the experimental group.

Histological assessment of grafts and lumen quantification. Following OMAG imaging, the hearts were perfused with PBS to wash out remaining intralipid solution followed by perfusion for 10 min with a 1:1 mixture of fluorescein-labeled or unconjugated GSL I (Vector Labs, 8 μg/mL) and rhodamine-labeled UEFA 1 (Vector Labs, 8 μg/mL) in order to label the ECs of perfused rat (GSL I-bound) and human (UEA 1-bound) endothelium. The hearts were flushed with PBS and then sliced into 2- to 3-mm-thick sections from the apex for paraffin processing and embedding34,35. Four-μm sections were cut and stained for picrosirius red/fast green (to assess infarct size) or subjected to immunohistochemistry31,18. Briefly, slides were de-paraffinized and rehydrated followed by enzymatic antigen retrieval (EAR) with proteinase k (Roche, 15 μg/mL in 10 mM Tris/HCl) at 37 °C for 20 min or heat-induced epitope retrieval (HIER) for 20 min in boiling Tris/EDTA buffer (pH 9.0) or citrate buffer (pH 6.0). The slides were blocked and permeabilized with natural donkey serum (Jackson, 10%) and 0.5% Triton X-100 followed by overnight incubation with primary antibodies: rabbit pAb to D2Red (Clontech 632496, 1:75, EAR) to detect TdTomato reporter in mTm-hESC-ECs, rabbit pAb to HCD31 (Cytomatrix 28364, 1:300, HIER Tris/EDTA buffer) to detect HSC-ECs, rabbit pAb to smooth muscle actin (abcam 7817, 1:100), goat Ab to GSL I (Vector AS-2104, 1:100), mouse pAb to human β-MHC (graft size), a biotintylated goat anti mouse secondary antibody (Jackson, 1:500) was used in conjunction with the Vectastain Avidin/Biotin Complex (ABC Kit) (Vector labs) and 3,3′-diaminobenzidine (Sigma) followed by routine hematoxylin counterstain to detect nuclei. The density and size of perfused lumens in vascular grafts were quantitated with custom Matlab code to analyze the confocal microscopic images of GSL I or UEA I. Please note that, while the lectins GSL I and UEA I are species specific in their endothelial-binding affinity, the antibodies against them were not. Anti-GSL I stains were used to evaluate perfused lumens of both species, while stains anti-β-MHC were used to detect perfused human vessels. The graft size was determined by total β-MHC+ area relative to total left ventricular histological area of 4-μm section containing 4-5 heart sections. The number of cardiomyocytes per area (cardiomyocyte density) was quantified by manual cell counting of β-MHC+ cells in >20 confocal microscopic images. Overall and cardiomyocyte-specific viability within the graft was determined by total nuclei or sarcromeric a-actinin+ cell colocalization with TUNEL+ marker determined by particle analysis of >40 confocal microscopic images on ImageJ. All analysis of animal experiments was performed by a blinded observer without knowledge of the experimental group.

Statistical analysis. Unless otherwise noted, single variable analysis with two-tailed t test assuming unequal variance was used to determine statistical significance between two samples. All results are presented as mean ± standard error and assumed to be distributed approximately normally. For in vitro μVs, the sample number represents the number of constructs analyzed unless otherwise noted. For in vivo experiments, the sample number per group represents the number of animals. Significance was defined as *p < 0.05 and **p < 0.01 for all results.

Code availability statement. All codes used in this study are available from the corresponding authors upon request, except the custom codes used for OMAG and OMAG-V acquisition and analysis are available from Dr. Rui Kang Wang (wangrk@uw.edu) upon request. The custom Matlab code for quantifying density and size of perfused lumens in vascular grafts has been deposited in the Gene Expression Omnibus database under accession code GSE124314.

Received: 19 October 2017 Accepted: 4 January 2019
Published online: 04 February 2019

References
1. MacNeil, S. Progress and opportunities for tissue-engineered skin. Nature 445, 874–880 (2007).
2. Atala, A. Engineering organs. Curr. Opin. Biotechnol. 20, 575–592 (2009).
3. Atala, A., Kasper, F. K. & Mikos, A. G. Engineering complex tissues. Sci. Transl. Med. 4, 161rv12 (2012).
4. Ogle, B. M. et al. Distilling complexity to advance cardiac tissue engineering. Sci. Transl. Med. 8, 342ps13 (2016).
5. Laffamme, Ma & Murry, C. E. Heart regeneration. Nature 473, 326–335 (2011).
6. Mozzaffarian, D. et al. Executive summary: heart disease and stroke statistics—2015 update. Circulation 131, 434–441 (2015).
7. Stevens, K. R. et al. Physiological function and transplantation of scaffold-free and vascularized human cardiac muscle tissue. Proc. Natl Acad. Sci. USA. 106, 16568–16573 (2009).
8. Fulloch, N. L. et al. Growth of engineered human myocardium with mechanical loading and vascular coculture. Circ. Res. 109, 47–59 (2011).
9. Sehine, H. et al. Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. Circulation 118, S145–S152 (2008).
10. Coulombe, K. L. K. & Murry, C. E. Vascular perfusion of implanted human engineered cardiac tissue. IEEE https://doi.org/10.1109/NBEC.2014.6972763 (2014).
11. Roberts, M. A. et al. Stromal cells in dense collagen promote cardiomyocyte and microvascular patterning in engineered human heart tissue. Tissue Eng. Part A 22, 633–644 (2016).
12. Sehine, H. et al. In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. Nat. Commun. 4, 1399 (2013).
13. Zhang, B. et al. Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. Nat. Mater. 15, 669–678 (2016).
14. Baiguera, S. & Ribatti, D. Endothelialization approaches for viable engineered tissues. J. Cardiovasc. Pharmacol. Ther. 19, 382–393 (2014).
15. Palpant, N. J. et al. Inhibition of catenin signaling respecifies anterior-like endothelium into beating human cardiomyocytes. Development 142, 3195–3205 (2015).
16. Palpant, N. J. et al. Generating high-purity cardiac and endothelial derivatives from patterned mesoderm using human pluripotent stem cells. Nat. Protoc. 12, 15–31 (2016).
17. Chong, J. H. et al. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primates hearts. Nature 510, 273–277 (2014).
18. Shiba, Y. et al. Human ES-cell-derived cardiomyocytes electrically couple and suppress arrhythmias in injured hearts. Nature 490, 322–325 (2012).
19. Wang, R. K., An, L., Francis, P. & Wilson, D. J. Depth-resolved imaging of capillary networks in retina and choroid using ultrahigh sensitive optical microangiography. Opt. Lett. 35, 1467–1469 (2010).
21. Chen, C.-L. & Wang, R. K. Optical coherence tomography based angiography [Invited]. Biomed. Opt. Express 8, 1056 (2017).
22. Zhang, Q. et al. Wide-field optical coherence tomography based microangiography for retinal imaging. Sci. Rep. 6, 22017 (2016).
23. Baran, U. & Wang, R. K. Review of optical coherence tomography based angiography in neuroscience. Neurophotonics 3, 010902 (2016).
24. Baran, U., Choi, W. J. & Wang, R. K. Potential use of OCT-based microangiography in clinical dermatology. Ski. Res. Technol. 22, 238–246 (2016).
25. Qin, W. et al. Depth-resolved 3D visualization of coronary microvasculature with optical microangiography. Phys. Med. Biol. 61, 7536–7550 (2016).
26. Gantz, J. et al. Targeted genomic integration of a selectable floxid dual fluorescence reporter in human embryonic stem cells. PLoS ONE 7, 1–9 (2012).
27. Mackie, A. R. & Losordo, D. W. CD34-positive stem cells: in the treatment of heart and vascular disease in human beings. Tex. Heart Inst. J. 38, 474–485 (2011).
28. Zheng, Y. et al. In vitro microvessels for the study of angiogenesis and thrombosis. Proc. Natl Acad. Sci. 109, 9342–9347 (2012).
29. Roberts, M. A., Kotha, S. S., Phong, K. T. & Zheng, V. Micropternning and assembly of 3D microvessels. J. Vis. Exp. https://doi.org/10.3791/54457 (2016).
30. Ramanathan, T. & Skinner, H. Coronary blood flow. Contin. Educ. Anaesth. Crit. Care Pain 5, 61–65 (2004).
31. Gerbin, K. A., Yang, X., Murry, C. E. & Coulombe, K. L. K. Enhanced electrical integration of engineered human myocardium via intracardiac versus epicardial delivery in infarcted rat hearts. PLoS ONE 10, e0131446 (2015).
32. Virag, J. I. & Murry, C. E. Myoфиbroblast and endothelial cell proliferation during murine myocardial infarction repair. Am. J. Pathol. 163, 2433–2440 (2003).
33. Wildemann, T. M., Mirhosseini, N., Siciliano, S. D. & Weber, L. P. Cardiovascular responses to lead are biphasic, while methylnitroxy, but not inorganic mercury, monotonically increases blood pressure in rats. Toxicology 328, 1–11 (2015).
34. Lloyd-Jones, D. M. et al. Differential control of systolic and diastolic blood pressure: factors associated with lack of blood pressure control in the community. Hypertension 36, 594–599 (2000).
35. Wang, R. K., Zhang, Q., Li, Y. & Song, S. Optical coherence tomography angiography-based capillary velocimetry. J. Biomed. Opt. 22, 066008 (2017).
36. Zhang, M. et al. Cardiomycyte grafting for cardiac repair: graft cell death anti-death strategies. J. Mol. Cell. Cardiol. 33, 907–921 (2001).
37. Asahara, T. Isolation of putative progenitor endothelial cells for angiogenesis. Science 275, 964–966 (1997).
38. Ivanov, K. P., Kalinina, M. K. & Levkovich, Y. I. Blood flow velocity in capillaries of brain and muscles and its physiological significance. Microvasc. Res. 22, 143–155 (1981).
39. Mayrovitz, H. N., Larnard, D. & Duda, G. Blood velocity measurement in human conjunctival vessels. Cardiovasc. Dis. 8, 509–526 (1981).
40. Arfors, K. E., Bergqvist, D., Intaglietta, M. & Westergren, B. Measurements of blood flow velocity in the microcirculation. Ups. J. Med. Sci. 80, 27–33 (1975).
41. Koffler, J. et al. Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. Proc. Natl Acad. Sci. USA. 108, 14789–14794 (2011).
42. Baranski, I. D. et al. Geometric control of vascular networks to enhance engineered tissue integration and function. Proc. Natl Acad. Sci. 110, 7586–7591 (2013).
43. Madden, L. R. et al. Proangiogenic scaffolds as functional templates for cardiac tissue engineering. Proc. Natl Acad. Sci. USA. 107, 15211–15216 (2010).
44. Chiu, L. Y. Y., Montgomery, M., Liang, Y., Liu, H. & Radisic, M. Perforable branching microvessel bed for vascularization of engineered tissues. Proc. Natl Acad. Sci. USA. 109, E3414–E3423 (2012).
45. Juhas, M., Engelmayr, G. C. J., Fontanella, A. N., Palmer, G. M. & Bursac, N. Biomimetic engineered muscle with capacity for vascular integration and functional maturation in vivo. Proc. Natl Acad. Sci. USA 111, 5508–5513 (2014).
46. Lammle, M. et al. Cardiomycytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat. Biotechnol. 25, 1015–1024 (2007).
47. Burridge, P. W., et al. Chemically defined generation of human cardiomycytes. Nat. methods. 11, 855 (2014).
48. Kim, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of human deletions, insertions and gene fusions. Genome Biol. 14, R36 (2013).
49. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169 (2015).
50. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140 (2010).
51. Liberzon, A. et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst. 1, 417–425 (2015).
52. Tomlins PH, Wang RK. Theory, developments and applications of optical coherence tomography. J. Phys. D: Appl. Phys. 38, 2519–2535 (2005).
53. Choi, W. J., Wang, H. & Wang, R. K. Optical coherence tomography microangiography for monitoring the response of vascular perfusion to external pressure on human skin tissue. J. Biomed. Opt. 19, 056003 (2014).

Acknowledgements
We acknowledge the Lynn and Mike Garvey Imaging Laboratory in the Institute of Stem Cell and Regenerative Medicine, the Nanotext User Facility, and Flow Cytometry Facility, all at the University of Washington. We thank Ms. Jun Xue and Mr. Daniel Lih for their help in endothelial cell and cardiomyocyte differentiation; and Dr. Lil Bapon and Dr. Hans Reinecke for helpful discussions along the course of these experiments. We acknowledge the financial support of National Institute of Health grants R01HL141570 (to Y.Z. and C.E.M.), DP2DK102258 (to Y.Z.), P01HL094374, R01HL128362, and P01GM081619 (to C.E.M.), R01EY024158 and R01HL093140 (to R.W.), T32HL7312 (to M.A.R., training grant), and T32E01650 (to N.Z., training grant). We also acknowledge the support of the Foundation Leeduq Transatlantic Network of Excellence (to C.E.M.).

Author contributions
C.E.M. and Y.Z. conceived the project; M.A.R., N.Z., C.E.M., and Y.Z. designed the experiments; M.A.R., N.Z., A.M., and Y.Z. performed both in vitro and in vivo experiments; M.A.R. and N.Z. analyzed the data; M.A.R., W.Q., and W.W. performed OMAG imaging and analysis; Y.W. and Y.Z. performed RNAseq analysis; M.A.R., N.Z., C.E.M., and Y.Z. interpreted the data and wrote the manuscript. R.K.W. oversaw the OMAG experiments; C.E.M. and Y.Z. oversaw all phases of the project and obtained research funding. All authors interpreted the data, edited, and approved the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-08388-7.

Competing interests: C.E.M. is a scientific founder and equity holder in CytoCardia. R.K. W. discloses intellectual property (US8180134 In vivo structural and flow imaging (2006), US9013555 Method and apparatus for ultrahigh sensitive optical microangiography (2009)) owned by the Oregon Health and Science University and the University of Washington related to OCT angiography, and licensed to commercial entities, which are related to the technology and analysis methods described in part of this manuscript. R.K.W. also receives research support from Carl Zeiss Meditec Inc, Moptim Inc, Facebook Technologies LLC, and Colgate Palmolive Company. He is a consultant to Insight Photonic Solutions, Kowa, and Carl Zeiss Meditec. All the other authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: Nature Communications thanks Wolfram Zimmermann and the other anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.