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Supplementary Information for

Mature vessel networks within engineered tissue promote graft-host anastomosis and prevent graft thrombosis

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Materials and Methods

Cell culture Cell culture Human umbilical vein endothelial cells (HUVECs) (Lonza, USA) and green fluorescent protein (GFP)-expressing HUVEC (HUVEC-GFP) (Angio-Proteomie, USA) were cultured in EGM-2, supplemented with EGM-2 SingleQuots (bullet kit, LONZA, USA) and 3% fetal bovine serum (FBS; HyClone, USA). Human normal dermal fibroblasts (HNDF; Lonza, USA) were cultured in Dulbecco's minimal essential medium (DMEM; Gibco® Life Technologies), supplemented with 10% FBS, 1% nonessential amino acids (Biological Industries), 0.2% β-mercaptoethanol (Gibco, Carlsbad, CA), and 100 units/ml penicillin/streptomycin (Pen-Strep Solution, Biological Industries, Israel). All cells were grown in 5% CO_2, at 37ºC in a tissue culture incubator (Thermo).

Scaffold preparation 0.8-1-mm-thick and 6mm-diameter porous sponges composed of 50% poly-L-lactic acid (PLLA) (Polysciences, Warrington) and 50% poly(lactic-co-glycolic acid (PLGA) (Boehringer Ingelheim) were fabricated utilizing a particle-leaching technique to achieve pore sizes of 212−600μm and 93% porosity(1).

Seeding procedures GFP-HUVECs ($5x10^5$) and HNDFs ($1x10^5$) were resuspended in 7μl of a 1:1 (v/v) mixture of 15U/ml fibrinogen (Sigma-Aldrich) and 50mg/ml thrombin (Sigma-Aldrich). The co-culture suspension was then seeded onto the scaffolds and allowed to solidify for 30-45 min, at 37°C, 5% CO_2. After solidification, a 1:1 mixture of modified EGM2:HNDF culture media was added to each well. Medium was replaced every other day.

Mouse dorsal skinfold window implantation All surgical procedures and animal studies were approved by the animal ethics committee at the Technion. The dorsal window chamber procedure was based on the Palmer et al. protocol, with minor modifications(2). Briefly, athymic nude mice (~30g, 7-9-weeks-old; Harlan Laboratories) were anesthetized via intra-peritoneal injection, with a mixture of ketamine-xylazine, at a dose of 100mg/kg and 10mg/kg, respectively. Thereafter, buprenorphine (0.05 mg/kg) was subcutaneously injected every 12 h, for two days. The dorsal region of the mouse torso and tail were cleaned with chlorhexidine solution and iodine to establish an aseptic working field. The two window frames were implanted by inserting 3 screws though the mouse's stretched skin and the frames were sutured to each other through the mouse skin, using 4-0 silk sutures. The forward-facing portion of skin was cut along the circumference of the window opening and removed, leaving the opposing layer intact. The window groove was washed with 37ºC pre-warmed saline, closed with a 13mm-diameter coverslip glass (Electron Microscopy Sciences, PA, USA), and fixed with a snap-ring. The mice were monitored daily to assess general health. Out of 108 mice which had dorsal skinfold window implantations, 6 (0.05%) showed infection symptoms within the first 48hrs post-implantation, and therefore were not included in the experiments. The sterile work under specific-pathogen-free (spf) conditions has decreased the probability for post- implantation infections.

Scaffold implantation Two or three days after dorsal window chamber implantation, mice were anesthetized as described above, or with 2% isoflurane. Following snap-ring and

coverslip removal, the window groove was washed with pre-warmed saline, and a scaffold was placed within the window groove. A new coverslip was placed and fixed with the snap-ring. Mice were monitored daily and subsequently assessed by intra-vital microscopy.

Intra-vital imaging Intra-vital microscopy was performed every 3-4 days for up to 14 days, using a Zeiss LSM510 META or LSM700 confocal microscope (Carl Zeiss). To visualize the host vasculature, Alexa-flour647 (AF647) anti-mouse CD31 (Biolegend) was intravenously injected to the mouse tail vein. The antibody was then allowed to circulate for 30 minutes before mice were anesthetized, as described above. Tetramethylrhodamine-isothiocyanate (TRITC)–dextran, (average MW 155,000, Sigma–Aldrich) was intravenously injected into the tail vein. Images were acquired using the Zen software provided with the microscopes. At the end of each experiment, mice were euthanized with CO_2, grafts were retrieved, fixed in 10% formalin (Sigma-Aldrich), and visualized using an LSM700 confocal microscope.

Whole-mount immunofluorescence staining Scaffolds were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences), for 15 min, rinsed with PBS (Gibco® Life Technologies), and permeabilized with 0.3% Triton X-100 (Bio Lab Ltd.), for 15 min at room temperature (RT). Following PBS washes and overnight incubation in blocking buffer (5% BSA (Merck Millipore) in PBS) at 4°C, scaffolds were incubated overnight at 4°C with primary antibodies: 1:50 mouse-anti-human αSMA (Dako), 1:100 mouse anti-human NG2 (Santa Cruz), 1:500 anti-collagen type IV (Sigma- Aldrich) or 1:100 goat anti-VE-cadherin (Sigma- Aldrich) diluted in blocking buffer. Following PBS washes, samples were incubated with secondary antibodies: 1:400 donkey anti-mouse Alexa Fluor®647 or 1:100 donkey anti-goat Alexa Fluor®647 (Jackson ImmunoResearch) and 1:1000 DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich), and DAPI (1:1000, Sigma-Aldrich), for 2h at RT. Following PBS washes, scaffolds were maintained in 0.5% PFA until they were imaged with a Zeiss LSM700 confocal microscope (Carl Zeiss), using the Zen software. Image processing and further analyses were performed using FIJI software.

Quantification of vessel network formation and development

Confocal images of the HUVEC-GFP vessel networks were acquired on days 1, 4, 7, 10 and 14 after seeding and days 1, 7, 10 and 14 after implantation inside the mouse dorsal window chamber. To analyze cluster formation, the average area of cell clusters was analyzed using in-house written matlab. Briefly, images were transformed into a binary image and the size of each element was measured using the regioprops function. Elements were then averaged. Average vessel length (mm) and total number of vessel junctions within the constructs were quantified using AngioTool software (National Cancer Institute). Longer vessel lengths and higher vessel junction counts were considered an indication of a more developed and complex network. Confocal images of neuron-glial antigen 2 (NG2) or alpha smooth muscle actin (αSMA)-stained scaffolds on days 1, 7 and 14 post-seeding were analyzed using the 3D IMARIS software (version 8.4.1, BitplaneInc.) and the surface algorithm to determine NG2 expression (volume; μm^3) and density of αSMA (%). A distance transformation algorithm was used to determine the distance (μm)

of αSMA-positive cells from the nearby GFP-HUVEC, and to create a color-coded map of distances ranging from 0-250μm.

Protein extraction and immunoblotting Scaffolds (triplicates) were washed with PBS and placed in a single tube containing RIPA solution: (1% sodium deoxycholate (DOC) (Sigma-Aldrich), 1% 100x-Triton (Biolab), 50mM Tris-HCl (Sigma- Aldrich), 150mM NaCl (Sigma-Aldrich), 5mM EDTA (Sigma- Aldrich)) with protease inhibitors cocktail (100mM PMSF, 2mg/ml leupeptin, 50mM Na-orthavanadate, 50mM aprotinin (Sigma- Aldrich)). Scaffolds were homogenized and the total protein content was determined using the Bradford method (Bio-Rad, Hercules, CA). Proteins were separated on a 4-12% SDS-PAGE gel (NuPage©, Life Technologies) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% non-fat milk (Bio-Rad, Hercules, CA), the membranes were immunoblotted (overnight, at 4oC) with primary antibodies: 1:300 rabbit anti-vWF (Abcam), 1:500 rabbit anti-αSMA (Abcam), 1:300 mouse anti-TF (Santa Cruz), 1:1000 mouse anti-GAPDH (Santa Cruz) and/or 1:500 mouse anti-CD31 (Dako) diluted in blocking buffer. The bound antibodies were detected following incubation with horseradish peroxidase-conjugated secondary antibodies with anti-mouse/rabbit IgG (GE Healthcare Life Sciences), followed by exposure to the enhanced chemiluminescence Western blotting system (ECL; Amersham Biosciences). Images were acquired with the LAS-3000 imaging system (FujiFilm). Dense cytometry analysis was performed using multi-gauge software (Bioz). Data are presented relative to either GAPDH or CD31 expression.

Evaluation of vessel shape Confocal images were transformed into binary images and each image was divided into separate elements using a self-written MATLAB algorithm. Eccentricity is a measurement of the ellipticity of an object. Round objects are scored 0 and as the shape becomes more compressed and elongated, scores rise to 100. Briefly, images were transformed into a binary image and eccentricity was measured by the applying imageprops function on the image, values were then divided into bins.

Tracking host vessel invasion into the scaffold Graft perimeter was determined, for each intra-vital image, using the bright-field channel. Using an in-house written MATLAB code, three circles of diameters of 6mm (graft perimeter), 3.75mm and 1.5mm were created to define three distinct regions: R1- the outer ring, R2- middle ring, and R3- center of the scaffold (Figure 3B). Then, host vessel presence was evaluated and their coverage (μm^2) of the three different regions was determined by counting number of vessels pixels in each region.

Graft blood perfusion To detect the presence of blood flow within the engineered blood vessel, mice were first anesthetized and then intravenously injected with 10 mg/ml TRITC–dextran, (average MW 155,000, Sigma–Aldrich) through the tail vein. The red dye was then viewed via intravital imaging.

Clot area within the grafts To determine clot accumulation, digital photos of the graft within the dorsal window chamber (Samsung) were analyzed by a blinded assessor. Dark red regions or any color change from the color of the mouse skin or area outside the implant, were manually marked and graft perimeter was also marked. Percentage of marked area within each graft was calculated using MATLAB, dividing number of clot area pixels by number of graft area pixels.

Immunohistochemical staining Grafts retrieved at 10 days or 2 weeks post-implantation and fixed for 15 min with 4% paraformaldehyde (PFA; Electron Microscopy Sciences). Following overnight incubation in a 30% (w/v) sucrose solution, grafts were embedded in optimal cutting temperature (OCT) compound (Tissue-Tec), and frozen for subsequent cryosectioning to 5-mm thick sections. Slides were rinsed twice in 0.025% Triton X-100 (Bio Lab) and incubated for 30min with 2.5% goat serum (Vector Labs) in RT. Following overnight incubation with 1:200 rat- anti-mouse CD40 (abcam) in 2.5% goat serum (Vector Labs) at 40c, slides were rinsed twice with 0.025% Triton X-100 (Bio Lab). The activity of endogenous peroxidase was quenched by incubating with 0.3% H2O2 in methanol for 15 min in RT. Slides were washed twice with 0.025% Triton X-100 (Bio Lab) and incubated for 30min with ImmPRESSTM - rat IgG-HRP (Vector Labs). Detection was done using ImmPACT DAB Peroxidase (HRP) Substrate (Vector Labs). Next, slides were stained with 0.1% Mayers hematoxylin (Sigma-Aldrich) for 2 min, followed with tap water rinsing Slides were dipped in double distilled water (DDW) and dehydrated by serial immersions in increasing concentrations of ethanol. Finally, slides were dipped in xylene and covered with Vectamount (Vector Labs). Slides were imaged with fluorescence microscopy (Zeiss). Images were acquired using the Zen software provided with the microscopes and analyzed using FIJI software.

Statistical Analysis Data are presented as mean ± SEM. Group differences were determined by a one- or two-tailed Student's t test. Where appropriate, data were analyzed by two-way ANOVA, Tukey's multiple comparison tests, or by one-way ANOVA, followed by Mann-Whitney's comparison tests. p values below 0.05 were taken to indicate a statistically significant difference between groups. Statistical analyses were performed using GraphPad Software.

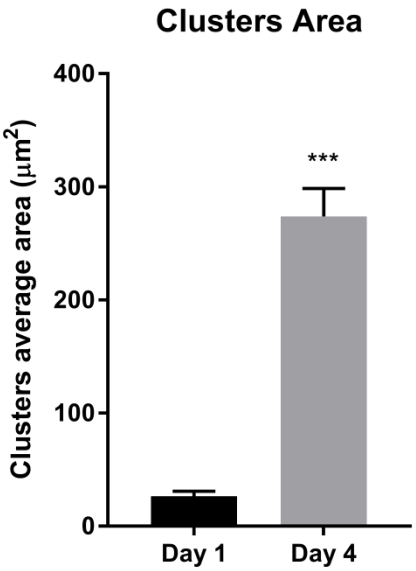

Fig. S1. cell cluster formation. 3D confocal images of scaffolds cultured for 1 day and 4 days in vitro. The average cell area was analyzed using in-house written matlab. Data are presented as means ± SEM, and were analyzed by student's t-test. $n \geq 3$ scaffolds per experiment. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

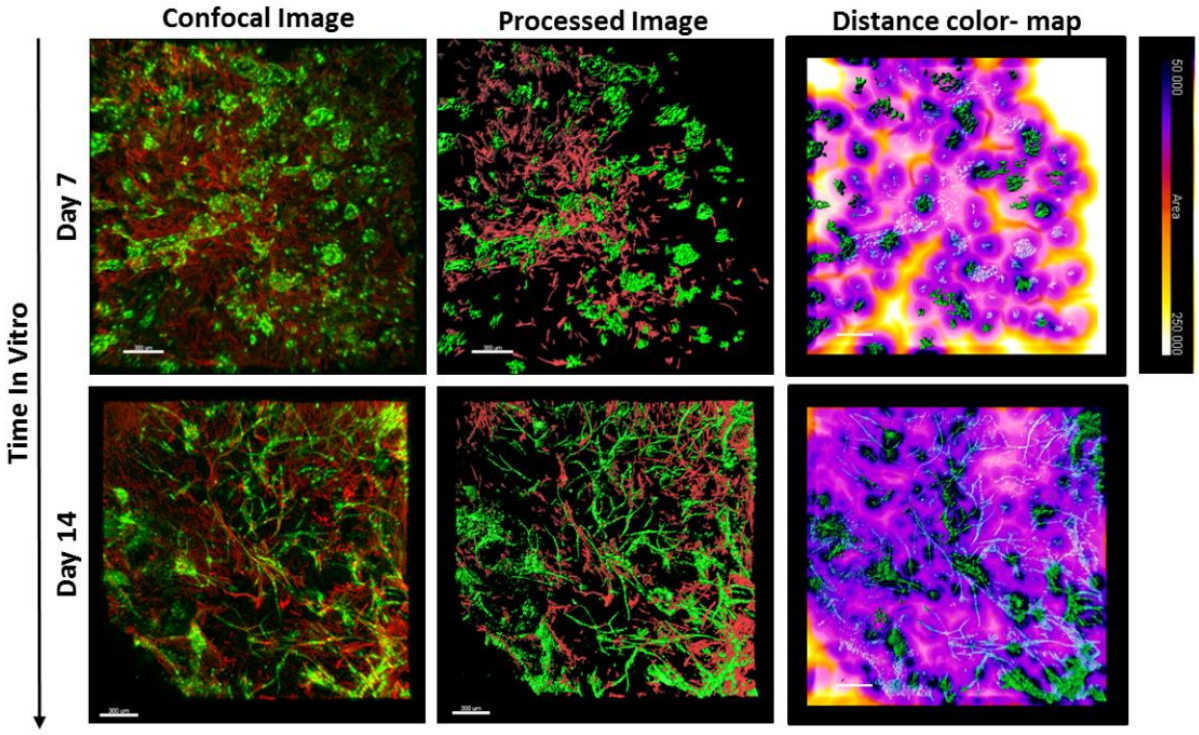

Fig. S2. The proximity of αSMA-positive cells to GFP-ECs. 3D confocal images of scaffolds cultured for 7 (upper panel) or 14 days (lower panel), were first processed using the Imaris surface rendering analysis (processed images column) and then analyzed with the distance transformation algorithm to measure the closest distance between each αSMA–positive cell (red)

and an EC (green). A color-coded map was generated, with blue representing close elements and white, distal elements. Scale bar= 300µmType or paste caption here.

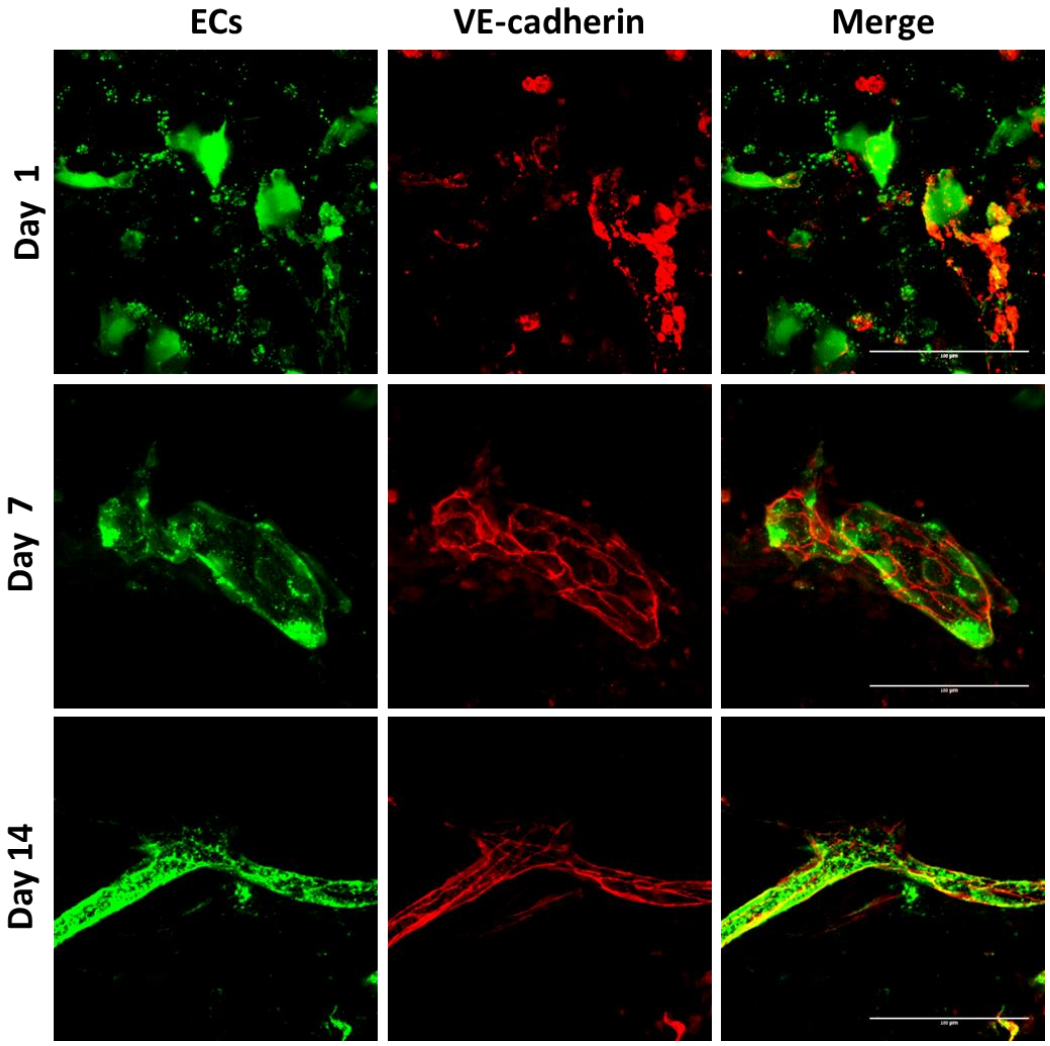

Fig. S3. Adherences junctions organization during engineered- vessel maturation. Representative confocal images of whole-mount immunofluorescent scaffolds on days 1, 7 and 14,

showing VE-cadherin (red) localization and expression in the forming vessel networks (green). Scale bar= 100µm

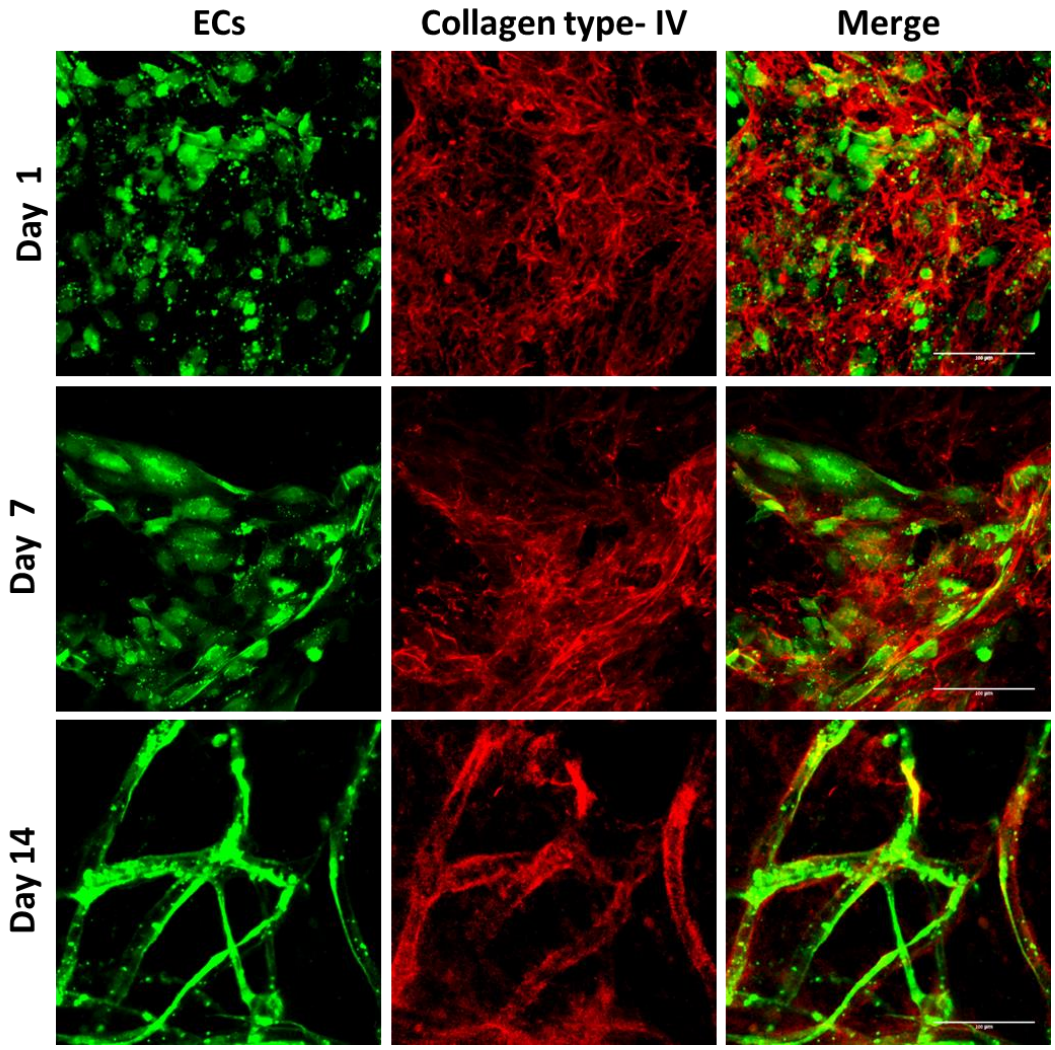

Fig.S4. ECM remodeling during engineered- vessel maturation. Representative confocal images of whole-mount immunofluorescent scaffolds on days 1, 7 and 14, showing type- IV

collagen (red) localization and expression in the forming vessel networks (green). Scale bar= 100μm

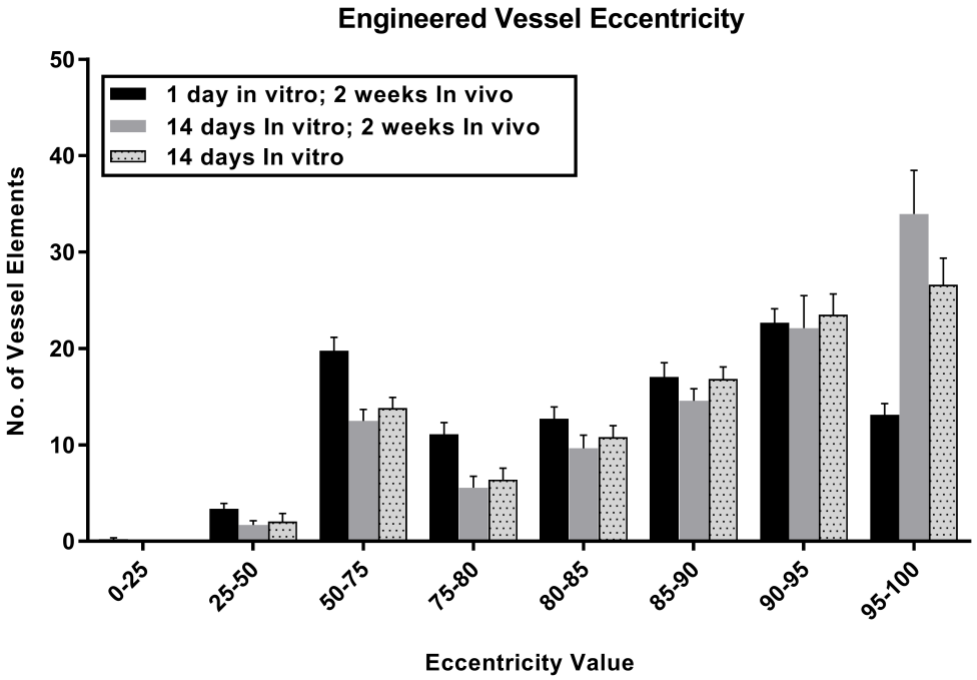

Fig.S5. Engineered- vessel eccentricity values. Eccentricity analysis of vessels in scaffolds seeded 1 or 14 days before implantation and grafted for 2 weeks, or of scaffolds cultured for 14 days in-vitro and imaged 1 day post-implantation. Eccentricity scores range from 0-100, with 0

indicating a round shape and 100 an elongated shape. Values were categorized into one of 6 bins (0-25, 25-50, 50-75, 80-85, 90-95, 95-100)

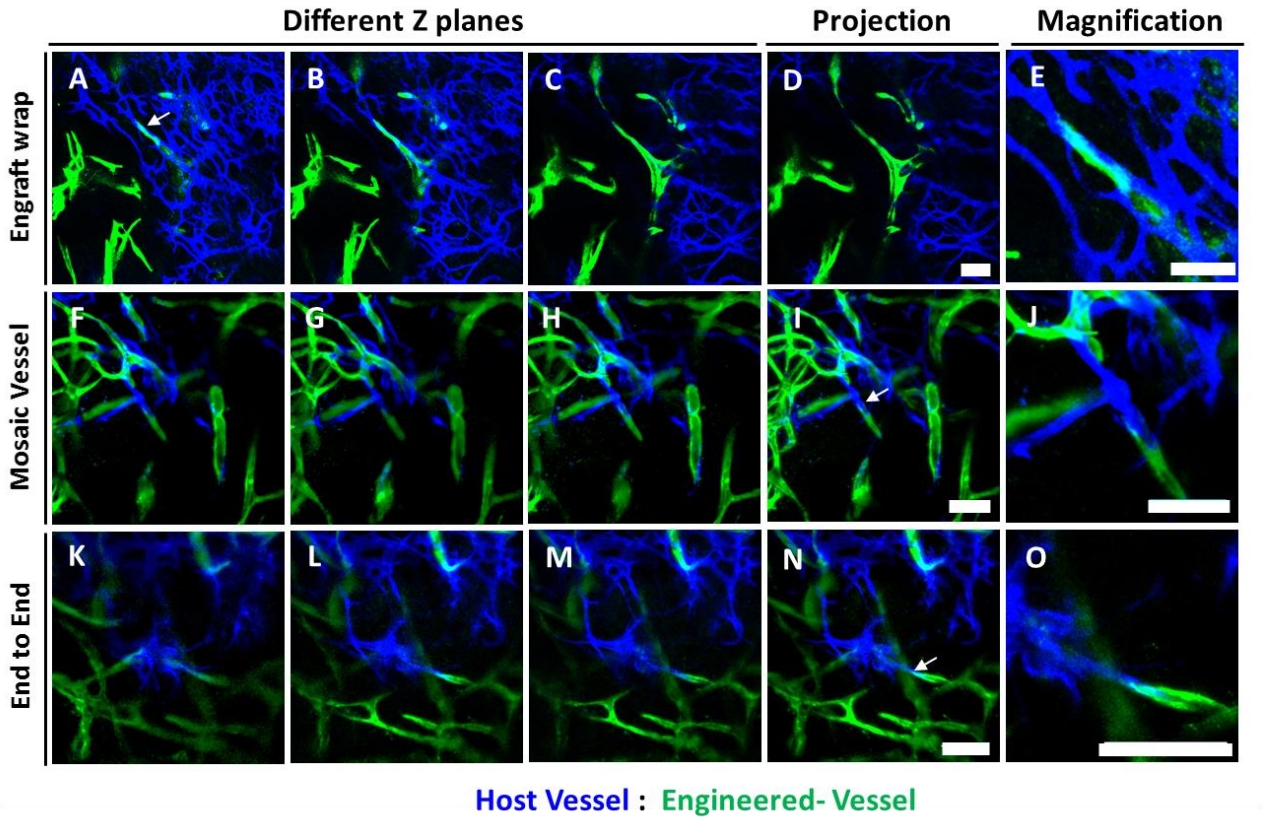

Fig. S6: Host-graft vasculature interactions. Intravital images showing various types of host-graft vessel interconnections: Engraft wrapping (4A-E), mosaic vessel formation (4F-J) and end-to-end connection (4K-O). Host vasculature is shown in blue (mCD31-AF647 was injected i.v. to visualize host vasculature) and grafted GFP-expressing HUVECs in green. To visualize vasculature orientation with respect to one another, different z planes (4A-C, 4F-H, and 4K-M) of the z-stack image projection are presented (4D, 4I, 4N, respectively). The white arrows indicate the area shown in the magnified images (4E, 4I, and 4O, respectively). Scale bar= 100µm.

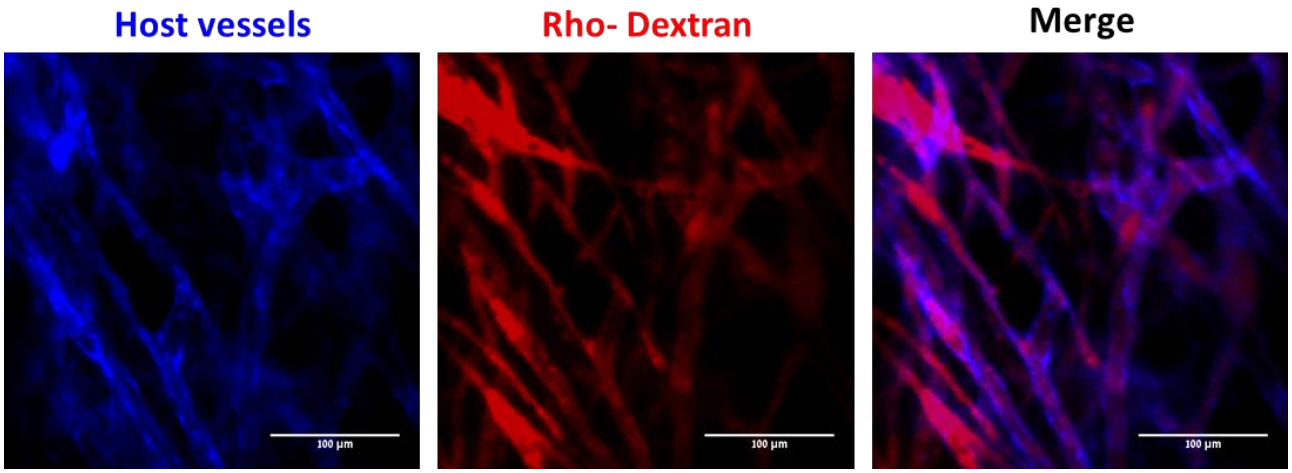

Fig. S7. Host vessel in- vivo staining. 3D confocal images of the invading host vessel into the scaffolds 10 days post- implantation. A specific mCD31-AF647 antibodies were injected into the mouse tail vein to visualize the host vasculature (blue). To mimic the blood flow, Rhodamine-

dextran (red) was injected into the mouse tail vein and used to visualize the vessel perfusion. Scale bar= 100µm

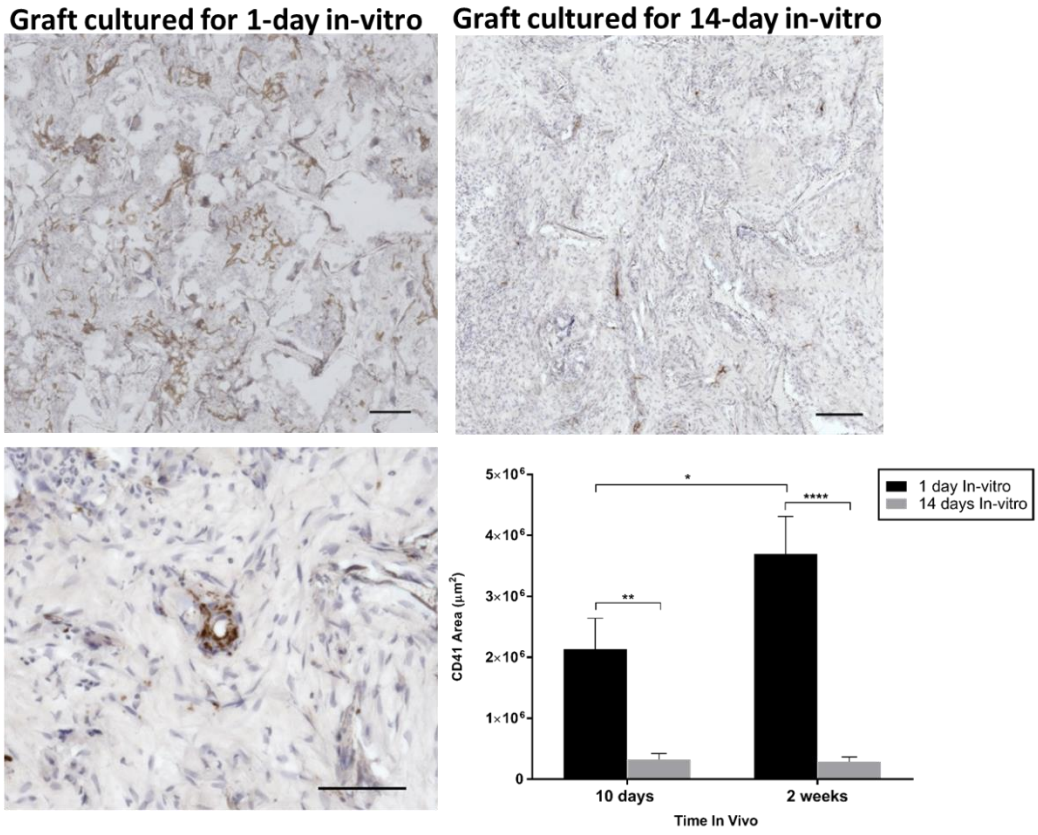

Fig. S8. Platelet aggregation in- vivo. Representative images of grafts cultures in- vitro for 1- day or 14 days and retrieved following 2 weeks of implantation. Immunohistochimestry staining of integrin alpha IIb (brown) and nuclei (blue). Integrin alpha IIb expression was determined by its area, and was analyzed using Fiji software. Data are presented as means ± SEM, and were analyzed

by one- way ANOVA followed by Tukey's multiple comparison tests. n ≥ 3 mice per experiment. (*p < 0.05, **p < 0.01, ****p < 0.0001). Scale bar= 200μm.

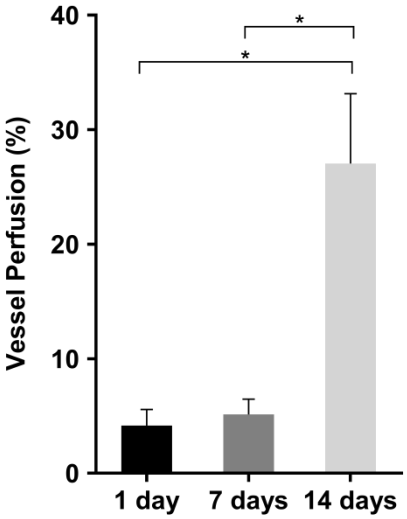

Figure S9. Vessel perfusion 14 day post implantation. Scaffolds that were cultured for 1, 7 and 14 days were implanted into a DWC model, and vessel perfusion was assessed at day 14 of implantation by the detection of Rho-dextran within the engineered- vessels. Data are presented as means ± SEM, and were analyzed by one-way ANOVA. n ≥3, *= p < 0.05.

Movie S1. Time-lapse video showing engineered vessel and host vessel perfusion on day 10 of graft implantation- GFP-HUVECs are green, host vessels are blue (mCD31-AF647 i.v.) and vessel perfusion is visualized by rhodamine-conjugated dextran (Rho-dextran,red), injected i.v.

References:

1. Lesman A, et al. (2011) Engineering vessel-like networks within multicellular fibrin-based constructs. *Biomaterials* 32(31):7856–7869.
2. Palmer GM, et al. (2011) In vivo optical molecular imaging and analysis in mice using dorsal window chamber models applied to hypoxia, vasculature and fluorescent reporters. *Nat Protoc* 6(9):1355–1366.