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Supplementary Information for

Implanted synthetic cells trigger tissue angiogenesis through *de-novo* production of recombinant growth factors

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This PDF file includes:

Other supplementary materials for this manuscript include the following:

Supplementary Text

Materials and Methods

Lipids

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), L-α-phosphatidylcholine (Egg, Chicken) (Egg-PC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC) were purchased from Lipoid (Ludwigshafen, Germany). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) was purchased from Avanti Polar Lipids (USA). Cholesterol was purchased from Sigma-Aldrich (Rehovot, Israel). DOPE-Cy5 was synthesized chemically by reacting DOPE to the NHS-Cy5 group (BDL pharma, China).

Preparation of S30-T7 bacterial lysate

S30-T7 bacterial lysate was prepared according to a previously reported protocol from *E. coli* BL21(DE3) cells (New England Biolabs, USA) transformed with pAR1219 vector (Sigma-Aldrich, Rehovot, Israel)(1).

Preparation of lipid phase for synthetic cells construction

Lipid mixtures were dissolved in chloroform (100mg/ml) and mixed in a glass vial at 1:1 or 4:3:1 weight ratios (elaborated in *SI Appendix*, **Table S2**). Then, 500µl mineral oil was added to each vial (Sigma-Aldrich, Rehovot, Israel). The oil mixtures were vortexed and then heated at 80°C for 1 hour to evaporate the chloroform. The lipid oil vials were stored at room temperature (RT) and used for up to two weeks.

Synthetic cells preparation using an emulsion transfer method

SCs were prepared according to the water-in-oil emulsion transfer method described previously with slight modifications (1). The process was conducted below 4ºC to preserve the molecular activity of the cell-free system. An internal solution based on a prepared S30-T7 bacterial lysate and its matching outer solution are listed in **supplementary table S5** (*SI Appendix*) and used for lipid composition studies only. The inner solution based on the PURExpress system (New England Biolabs, Massachusetts, United States) was supplemented with 10% (v/v) sucrose and 3% (w/v) polyethylene glycol (PEG)-6000 to enable proper encapsulation in SCs (*SI Appendix*, **Fig. S4)**. A suitable outer solution was prepared according to **supplementary table S6** (*SI Appendix*). These solutions were used for the rest of the experiments.

Flow cytometry analysis of synthetic cells

SCs were incorporated with a GFP-encoding DNA template and incubated at 37 ºC for 3.5 hrs to allow protein expression. The fluorescent signal produced by the particles was analyzed using a flow cytometry instrument (BD LSR-II– Digital, BD Biosciences, San Jose, CA, USA) with a high throughput system (HTS). 488nm laser and 530 ± 30 nm filter were used for excitation and emission, respectively. Ten thousand events were collected for each analyzed sample. Prior to the analysis, SCs were diluted 100-fold with phosphate-buffered saline (PBS). Then, they were filtered through a 70 µm cell strainer (BD Biosciences, San Jose, CA, USA) to eliminate cell aggregates. Data analysis was obtained using the FCS Express software. SCs without DNA templates were used to determine the GFP gate that allowed ~1% background noise. All samples were normalized to the mean value of the POPC-based GUV samples.

Percentage of active synthetic cells quantification

SCs were analyzed using the imaging flow cytometry AMNIS ImageStream®^{X} Mk II instrument (Luminex Corporation, USA) to determine the percentage of active SCs from the whole SC population. The SCs were incorporated with a GFP-encoding DNA template and detection of their activity (protein production capability) was based on fluorescence signal measurements. SCs without a DNA template were served as a negative control used to place the GFP positive events gate. Before analysis, the SCs were incubated at 37ºC for two hours and then diluted five-fold in PBS. The SCs were manually detected through the obtained images in the brightfield channel. Then, the intensity of fluorescence expression was assessed according to the measured values in the FITC channel (488nm laser and 505-560nm emission). Analysis was based on n=420 events of SCs with DNA and n=278 events counted for SCs without DNA template.

DNA vector design and purification

The DNA template encoding to super-folder GFP (sfGFP) was purchased from Sandia BioTech (Albuquerque, NewMexico, USA). This DNA template was cloned into a pET9a while adding a histidine×6 tag (elaborated in detail in a previously performed study by our group)(2).

The gene sequence encoding to human bFGF (based on the insert of PWPI_SPBFGF plasmid #25812, Addgene) was optimized according to the codon preference of *E. Coli* (Bio basic Canada Inc.). Then, the protein sequence was modified with two site direct mutations - C78S/C96S and fused to Thioredoxin and polyhistidine tags. The synthetic DNA sequence was amplified in a polymerase chain reaction (PCR) and cloned into a pET28A vector using NcoI/XhoI cleavage sites under the T7 promoter and terminator control. The ligation products were transformed into Dh5α *E. coli* strain (New England Biolabs, USA) by chemical transformation, and the proper construction of the vectors was tested in colony PCR using T7-promoter and terminator primers. The plasmids were purified from the selected colonies, and their sequences were then confirmed by a sequencing

service (Hylabs, Israel). All protein and primer sequences used are listed in **supplementary tables S3 and S4** (*SI Appendix*).

Protein expression and purification

BL21(DE3) *E. coli* cells were heat-shocked and transformed with the synthesized pET28A TRX-his-hbFGF vector. The transformed culture was incubated overnight at 37°C and 250rpm with 25µg/ml kanamycin in a 5-ml Luria-broth (LB) starter. The overnight culture was then inoculated into fresh Terrific-broth (TB) medium (in 1:100 volume ratio, 500 ml total volume), supplemented with 25µg/ml kanamycin. When optical density (OD,600nm) reached ~0.6, the cells induced 0.8mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to trigger protein expression. Following induction, the cells were grown to OD600 ~4 and harvested using centrifugation at 7000xg for 10 minutes (4°C). The bacterial pellet was re-suspended in a filtered PBS, and the cells were homogenized by two passes through a pre-cooled homogenizer (Avestin, Germany) or when a change in solution lucidity was detected. The solution was then centrifuged two times at 24,000xg for 15 minutes to separate the soluble phase from the non-soluble one. The desired protein was purified from the soluble fraction of cytoplasmic proteins on a HisTrap FF 5ml column (GE Healthcare, USA) using an AKTA pure chromatography system (Cytiva, USA). 20 and 500mM of imidazole in PBS solutions were used as binding and elution buffers, respectively. Following the purification process, samples of the elution peak were mixed and dialyzed at 4°C for a few rounds against PBS in a 12-14 kD membrane (Spectrum Laboratories, USA). Samples taken before and after the AKTA process were loaded onto a 12% SDS-PAGE gel to confirm protein purification. The gel was stained with Coomassie blue for 30 minutes and then washed and distained overnight. As expected, the ~35kDa expressed Trx-his-bFGF protein was observed in proximity to the 35kDa marker (*SI Appendix*, **Fig. S4**).

Size and concentration analysis of synthetic cells

ImageStream®X Mark II (Amnis Corporations, Seattle, WA) was used to measure the mean size and concentration of SCs with FGF-encoding DNA. The particles were prepared with DOPE-Cy5 (0.024% mol/mol) incorporated into their membrane and the DNA was stained with Hoechst (1:150, 1mg/ml stock, Sigma Aldrich, Rehovot, Israel). Before measurements, the SCs were diluted 5-fold in PBS and filtered through a 70µm cell strainer. Unstained SCs were used to set the fluorescence signal gates. 405nm laser with 435-505nm filter and 642nm laser with 642-745nm filter were used to identify Hoechst and Cy-5 signals, respectively. Analysis was performed using the IDEAS analysis software (Amnis, Seattle, WA). The diameter distribution of the SCs was estimated based on the bright field signal of a single SCs population that was positive to both Hoechst and Cy5. The concentration of the same SCs population was calculated by dividing the number of its events (n=292) by the total analyze*d* volume. This method was also used as part of the release mechanism

study of protein (GFP) from SCs. GFP expressing SCs were prepared with Cy5-labled lipid incorporated into their membrane. Samples of the same SC batch were measured using imaging flow cytometry at three different time points (0, 4, and 24 hours) after incubation in physiological conditions. The mean GFP fluorescence intensity per SC was calculated from the Cy5 labeled GFP expressing-SCs population. The same gates were applied for all samples based on unstained SCs control. The mean diameter of Cy5 labeled GFP expressing cells was calculated based on the bright field signal.

Quantification of FGF concentration in synthetic cells and reaction kinetics analysis

Western blot analysis was used to detect and quantify FGF production in SCs. The SC samples were incubated at 37°C and diluted 5-folds in ultrapure water before running them in 12% SDS-PAGE gel. For quantifying FGF concentration produced in SCs, we performed 3 hrs incubation. For reaction kinetics analysis, we incubated the SCs for 24 hrs, and samples of the same batch were collected over time and prepared later for loading into the same gel. Following electrophoresis, gels were blotted onto nitrocellulose membranes and blocked with a blocking buffer composed of 5% nonfat dry milk in Tris-buffered saline. Then, overnight incubation at 4ºC with primary antibody was executed with anti-hbFGF antibody (rabbit source, Abcam, Cambridge, UK) diluted 1:1000 in Tris-buffered saline with 0.5% Tween-20 and 0.5% nonfat milk powder. After the first antibody was washed, we incubated the membranes for 1 hr with anti-rabbit horseradish peroxidase-conjugated secondary antibody (goat origin, GenScript, New Jersey, USA) diluted 1:5000. Then, the membranes were washed and applied with a Forte western HRP Substrate (Merck, Germany). The chemiluminescence signal was detected using the FUSION FX-6 Spectra imaging system (Vilber, France). Band area analysis was performed using the ImageJ gel analysis plugin. FGF concentration in SCs was determined based on a purified TRX-FGF protein calibration curve. For reaction kinetics analysis, values were normalized relative to the mean value of 24 hrs time point samples. Both analyses included subtracting the non-producing SCs band area as a background "noise."

Cell culture

All cells were cultured at 37°C in a humidified atmosphere and 5% CO_2. Cell medium was exchanged every 2-3 days.

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and provided by Prof. Gera Neufeld (Cancer Research Center, The Bruce Rappaport Faculty of Medicine, Technion) and used up to passage 9 for proliferation assays and phosphorylation experiments. HUVECs were grown on gelatin-coated plates in M-199 medium supplemented with 20% (v/v) of fetal bovine serum (FBS), 1% (v/v) Penicillin-Streptomycin solution (100 1U/ml of Penicillin G Sodium Salt and

100 µg/ml of Streptomycin Sulfate) (Pen-Strep), 2mM L-Glutamine (Biological Industries, Beit Haemek, Israel) 0.5% (v/v) amphotericin B and 1% (v/v) vitamins (Sartorius, Beit Haemek, Israel). Purified FGF was added to the cell medium in a final concentration of 10 ng/ml (ab9596, Abcam, UK).

GFP-expressing HUVECs (Angio-proteomie, USA) and Dental Pulp Stem Cells (DPSCs, Lonza, Switzerland) were used for the rest of *in-vitro* experiments. GFP-expressing HUVECs were cultured in endothelial cell medium (ECM), supplemented with 5% FBS, 1% Endothelial cells growth supplement (ECGS), and 1% Penicillin solution (ScienceCell, USA) and used up to passage 8. DPSCs were cultured in low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco, Irland), supplemented with 10% FBS (Hyclone, USA), 1% non-essential amino acids (Gibco, Irland), 1% GlutaMAX (Gibco, Irland), and 1% penicillin-streptomycin-nystatin solution (Sartorius, Beit Haemek, Israel).

Activation of ERK1/2 in HUVECs by FGF-producing cell-free reaction

Primary HUVECs were seeded at a density of $1x10^5$cells/ml on a gelatin-coated six wells plate and grown in M-199 for 48 hours. Two hours before treatments were added, the media was replaced with fresh starvation media (0.25%FBS). The cells were then treated for 15 minutes with cell-free reactions based on SCs internal solution with or without TRX-FGF DNA (*SI Appendix*, **Supplementary table S5**, internal solution) that were pre-incubated for 2 hours in 37°C (800rpm), with purified TRX-FGF treatment or without treatment. FGF concentration was estimated as 50ng/ml. To prepare the cell extracts, HUVECs were washed with ice-cold PBS and lysed on ice for 20 minutes with RIPA buffer enriched with Phosphatase (Roche, Switzerland) and protease (Merck, Germany) inhibitors. Following incubation, the cell lysates were centrifuged for 15 minutes at 15000xg (4°C). Supernatants containing identical protein amounts were prepared according to the BCA assay for immunoblotting. Western blot procedure was used in the same manner described above with 5% BSA in TBST (Tris-buffered saline, 0.1% Tween 20) blocking buffer. To study the activation of MAPK signaling, membranes were incubated overnight with 1:200 anti-pERK1/2 (sc-7383, Santa Cruz Biotechnology, USA) primary antibody, washed and then treated with 1:5000 anti-rabbit horseradish peroxidase-conjugated secondary antibody. After quantifying the chemiluminescence signal, membranes were stripped, reprobed with 1:200 anti-total ERK1/2 (sc-514302, Santa Cruz Biotechnology, USA), and the rest of the process was performed similarly. Analysis was performed using the ImageJ software with the gel analysis plugin. The pERK/ERK band area ratio was calculated for each membrane and normalized relative to the ratio of no treatment control. βeta-actin /GAPDH were used as housekeeping genes.

Proliferation assays

PrestoBlue assay

Presto-blue viability assay was used to assess the activity of purified TRX-FGF compared with commercial bFGF in different concentrations and to test the mitogenic activity of the proangiogenic SCs. In both studies, primary HUVECs were seeded at 10,000 cells/well in complete M199 medium on gelatin-coated 96 well plates without adding FGF to their media. After the cells were attached to the plate surface, media was replaced with fresh media supplemented with the desired treatments. For comparison between the in-house purified TRX-FGF and a commercial bFGF (ab9596, Abcam, UK), the proteins were applied in concentrations of 0-100ng/ml. For assays with SCs, treatments of FGF expressing SCs (after estimated protein concentration of 50ng/ml, *SI Appendix*, **Fig.S7**), SCs lacking a DNA template (which served for studying the effect of the remaining reaction components), and untreated cells control were applied. Forty-eight hours later, cell proliferation was assessed using PrestoBlue assay (Invitrogen, USA) according to the manufacturer's protocol. The samples' fluorescence signal (560/590nm) was measured in an Infinite 200 PRO plate reader (Tecan, Austria). For purified proteins analysis, all samples were normalized to 100ng/ml TRX-bFGF treatment, and for proliferation assay, with SCs, all treatments were normalized to untreated cells control. M-199 only measurements were averaged and subtracted from all measuring values. Measurements in T=0 hours of at least five representative wells were done to verify no seeding variations.

Cell counting using high throughput microscopy

HUVECs were cultured and treated as described for the PrestoBlue assay with SCs. 48 hrs after treatment addition, cells were stained using Hoechst (1µg/ml, Sigma Aldrich, Rehovot, Israel) to mark cell nuclei. Nine fields in each well were scanned (10X) through the GE InCell analyzer 2000 instrument (GE Healthcare, USA) in the DAPI (4′,6-diamidino-2-phenylindole) and brightfield channels. Cell nuclei counting was obtained using the high content analysis software InCell Investigator. All values were normalized to the untreated control group.

Evaluation of three-dimensional vascular network formation on collagen-based scaffolds

HUVEC-GFP ($0.5x10^5$) and DPSC ($1.5x10^5$) were co-seeded in a small volume of M-199 medium (7µl) onto collagen-based CellGro scaffolds (provided kindly by Orthocell, Australia) and incubated for 15 minutes. After incubation, the constructs were transferred gently into 12 wells plate containing the same treatment groups elaborated in the proliferation assays section. Medium with fresh treatments was replaced every 2-3 days to maintain HUVECs viability and activate vascular network formation (results of a single-treatment experiment are presented in *SI-Appendix*, **Fig.S9**). Three-dimensional images of the scaffolds were taken using a confocal microscope (LSM700, Zeiss) with the 488nm laser on day seven of the experiment. Then, they were analyzed using AngioTool software (National Cancer Institute) to quantify the average vessel length and the total

number of vessel junctions within the constructs. Values were all normalized to untreated cell control.

Whole-mount immunofluorescent staining

Scaffolds (on day seven of the experiment) were fixed with 4% paraformaldehyde for 20 minutes, washed with PBS (Gibco, USA), and then permeabilized using 0.3% Triton X-100 (Bio Lab Ltd., Jerusalem, Israel) in PBS for 20 minutes. After rinsing with PBS, constructs were blocked with 5% bovine serum albumin (BSA, Merck Millipore, USA) in PBS for 1 hour. Then, scaffolds were incubated overnight (4°C) with 1:100 goat anti-VE-cadherin (sc-6458, Santa Cruz, USA) in addition to 1:500 mouse anti-collagen type IV (C1926, Sigma- Aldrich, Rehovot, Israel) or 1:100 mouse anti-human αSMA (M0851, Dako, Denmark) primary antibodies diluted in blocking buffer. Following PBS washes, scaffolds were treated with 1:100 donkey Cy3-conjugated anti-mouse and 1:100 donkey Cy5-conjugated anti-goat secondary antibodies (Jackson Immunoresearch Laboratory, USA) and 1:1000 DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, Rehovot, Israel) for 1 hour (RT).

Confocal microscopy imaging

A spinning disk confocal microscope (Nikon, Japan) was used to observe protein production and release in SCs. GFP-expressing SCs were prepared and incubated for 2 hours at 37 ºC to trigger protein expression. A sample of SCs without a DNA template was used as a negative control. All samples were incorporated with DOPE-Cy5 lipid in their outer lipid phase to mark the SC membrane. Before imaging, the samples were accordingly diluted 5-fold in a basement-membrane-extract solution (3445-005-01, R&D systems, USA) or 1% agarose in PBS (Bio-Rad, USA) and loaded into an optical µ-slide (Ibidi, Germany). The FITC channel was used to detect GFP production in SCs, while the AF647 channel was used to recognize the rhodamine labled SC membranes. The SCs were imaged through different z planes to identify the distribution of the produced protein within the cell and the mechanism of protein release. Confocal microscope LSM 710 (Zeiss, Germany) was used to demonstrate the proliferation assay results and evaluate vessel network formation in scaffolds. For the proliferation assay imaging, primary HUVECs were stained at the assay endpoint with Calcein-AM (2µmol/L, Sigma-Aldrich, Rehovot, Israel) and Hoechst (1µg/ml, Sigma Aldrich, Rehovot, Israel). The acquisition was performed using the ZEN software with 405 and 488nm lasers. To examine the stabilization of vascular networks in scaffolds, whole-mount immunofluorescent staining was applied. Constructs were imaged through 11 z-plane sections (total 20.415µm, 20x magnification, three random images per construct). The total Collagen-IV volume secreted in each scaffold was quantified by 3D IMARIS surface rendering analysis (version 8.4.1, BitplaneInc.).

Cytokine release profile

According to the manufacturer's instructions, the cytokine profile of conditioned media of vascularized scaffolds collected on day seven was determined using the Cytokine Human Angiogenesis Antibody Array (ab134000, Abcam, UK). Data were acquired using the FUSION FX-6 Spectra imaging system (Vilber, France) and analyzed using the Image-J gel quantification plugin. All values were normalized to untreated constructs control.

Immunohistochemical analysis

Immediately after extraction of Matrigel plugs, plugs were fixed with 3.5% formalin for 24 hours and then transferred to 70% EtOH before embedding in paraffin and sectioning. Some of the embedded slide sections were stained with hematoxylin-eosin (PathoLab, Ness Ziona, Israel), and some were used for immunohistochemical analysis. Plug paraffin sections were immunostained using a previously elaborated protocol (3) with rabbit anti-CD31 (1:2000) primary antibody (ab182981, Abcam, UK) incubation overnight (4°C). After rinsing slides in DW 3 times and blocking endogenous peroxidase activity with 0.3% hydrogen peroxide solution, the slides were washed in DW and incubated for 40 minutes at RT with ready-to-use secondary goat horseradish peroxidase (HRP) conjugated anti-rabbit antibody (MP7451 kit, Vector Laboratories). For color development, slides were rinsed in DW and then incubated with DAB solution (SK4105 kit, Vector Laboratories) for 1 minute, washed in DW, and counterstained with hematoxylin. All slides were scanned using the 3DHistech Panoramic 250 Flash III automated slide scanner (3DHistech, Hungary), and CD31 positive cells and vessel analysis were performed using the color deconvolution tool (ImageJ software) with H DAB vector. The images were adjusted to the same threshold value each time to calculate either CD31 positive cells, blood vessels, or the whole tissue area. To calculate the percentage area of CD31 or vessels, their measured area value was divided by the entire tissue area of the same image. All values were normalized to the vehicle-only (PBS) treatment that served as control.

Fluorescent staining of Matrigel plug-cryosections

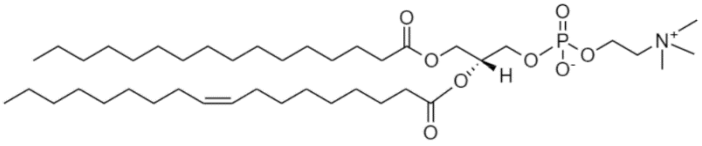
Rhodamine labeled 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine **(**DOPE) lipid was incorporated into SCs (0.02% mol/mol) to detect their distribution and remanence within the injected gel plugs. For this purpose, plugs were removed from mice 24 hours post-injection, embedded in O.C.T solution (Scigen, USA), and stored at -80°C. 10 µm thick sections were created using Leica CM1950 cryostat (Leica Biosystems GmbH, Germany). For fluorescence staining, the slides were fixed with 4% paraformaldehyde for 10 min on ice and washed in PBST (PBS with 0.025% Triton X-100). Then, DAPI nuclei fluorescent staining was used together with mounting solution (Bio-

Legend, USA), and the slides were closed with a coverslip glass. Slides imaging was performed with Leica DMI8 inverted fluorescent microscope (Leica Biosystems GmbH, Germany) with the DAPI and Texas-Red channels (*SI Appendix*, **Fig. S13**).

Hematology tests

Immediately after mice were sacrificed, whole blood samples were collected using a heparin-coated syringe into EDTA-covered vials. Blood cell count was measured (American Medical Laboratories, Israel) to study systemic immunogenicity and inflammatory effects of SCs administration. All values were normalized to vehicle-only (PBS) control.

1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
(POPC)



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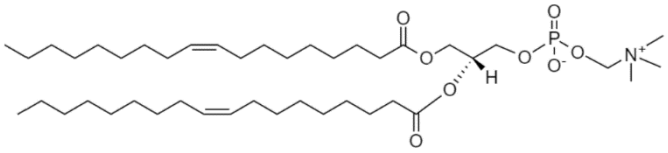
(DOPC)



L-α-phosphatidylcholine (EPC) *

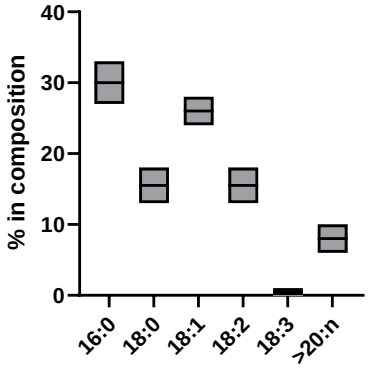


Figure S1. Schematics of the used phospholipids. *EPC is a mixture of naturally-derived phospholipids from egg yolk. The floating bar (min to max) graph describes the mixture composition distribution [%]. The number under each bar represents fatty acid tail length: saturation level (data presented according to Lipoid, E 80 SN datasheet).

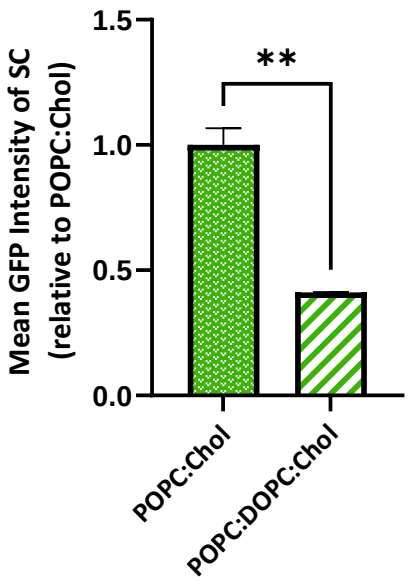


Figure S2. Effect of membrane composition on SC activity. Synthetic cells of POPC:Chol and POPC:DOPC:Chol were encapsulated with an adjusted PURE expression system and encoded to express GFP under physiological conditions. Their protein expression capabilities were studied using flow cytometry and presented as the mean GFP fluorescence intensity per single SC in each formulation. Values were normalized to POPC:Chol formulation. Chol=cholesterol. Data represent the mean ± SD (n=2 independent samples); Unpaired t-test P-value; **P=0.0065.

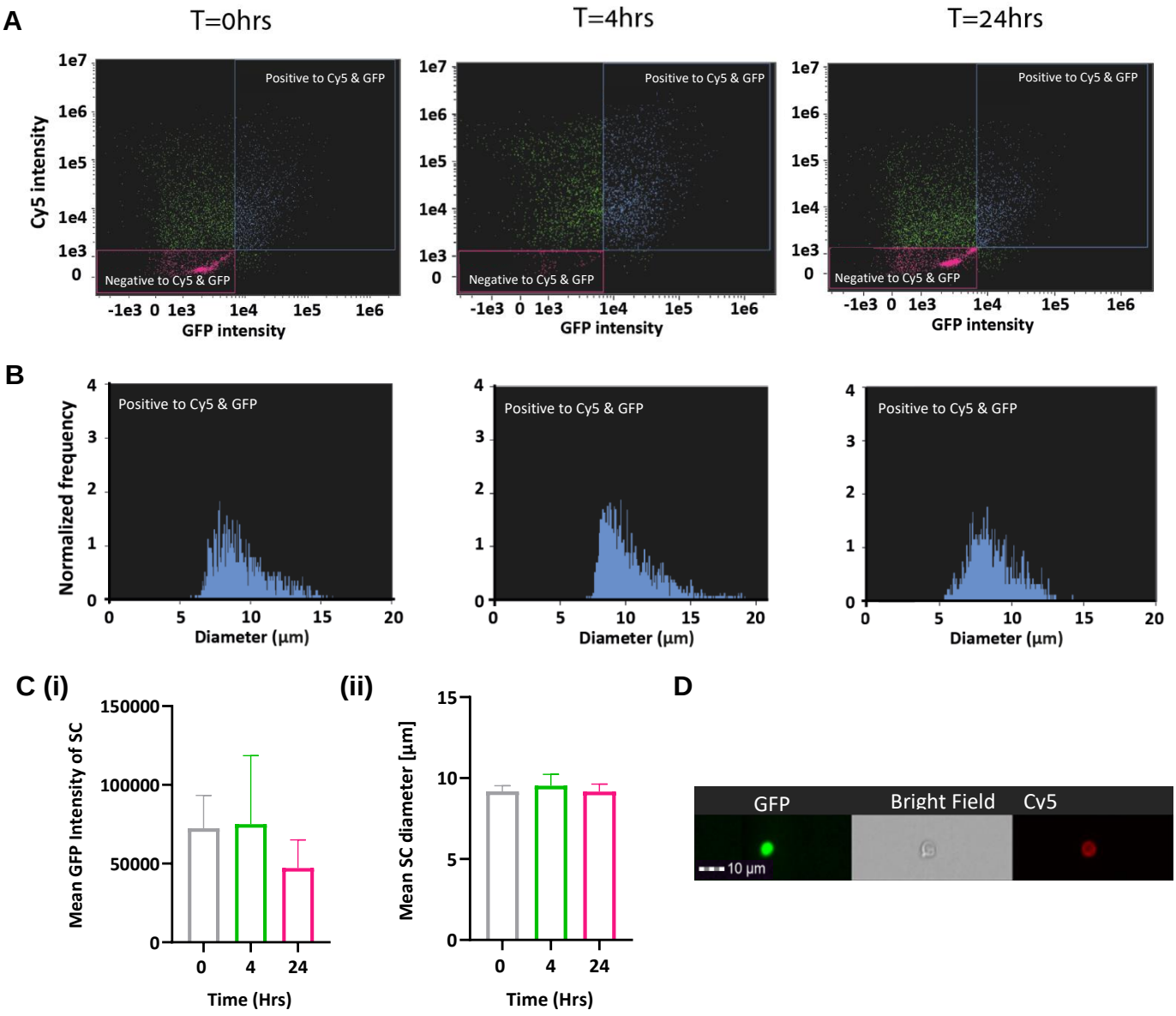


Figure S3. Imaging flow cytometry analysis of GFP-expressing SCs over 24 hours under physiological conditions. (A) Representative Cy-5-GFP intensity dot plots of Cy5 labeled GFP-expressing SC sample at three different time points (0, 4, 24hrs) and **(B)** the compatible size distribution histogram (diameter [µm], calculated based on the bright field signal) of the Cy5-GFP positive SC population. Time refers to 0 hours representing the experiment's starting point after incubating the SCs at 37°C for 2 hours (a sufficient incubation time for detecting GFP production). The samples were stored in physiological conditions throughout the whole experimental study. **(C) (i)** Column bars showing the mean GFP intensity per SC and **(ii)** the mean diameter of Cy5 labeled GFP-expressing SCs at the three measured time-points. Data represent mean ± SD (n=2-3 independent samples). **(D)** Representative image of GFP, Cy-5 positive SC collected using the AMNIS ImageStream®^{X} instrument.

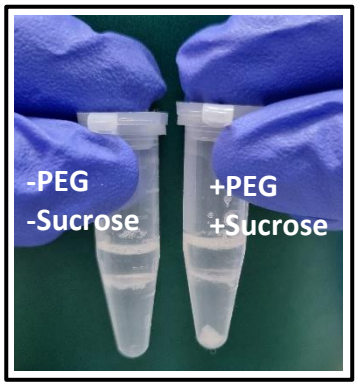


Figure S4. Adjustment of commercial CFPS system to proper performance in SCs. The PURExpress system was enriched with 10% (v/v) sucrose and 3% (w/v) polyethylene glycol (PEG)-6000 to enable SCs sedimentation using the water-in-oil emulsion transfer method. The osmolarity of the outer feeding solution was adjusted to equalize the value measured for the inner solution to prolong the particles' stability.

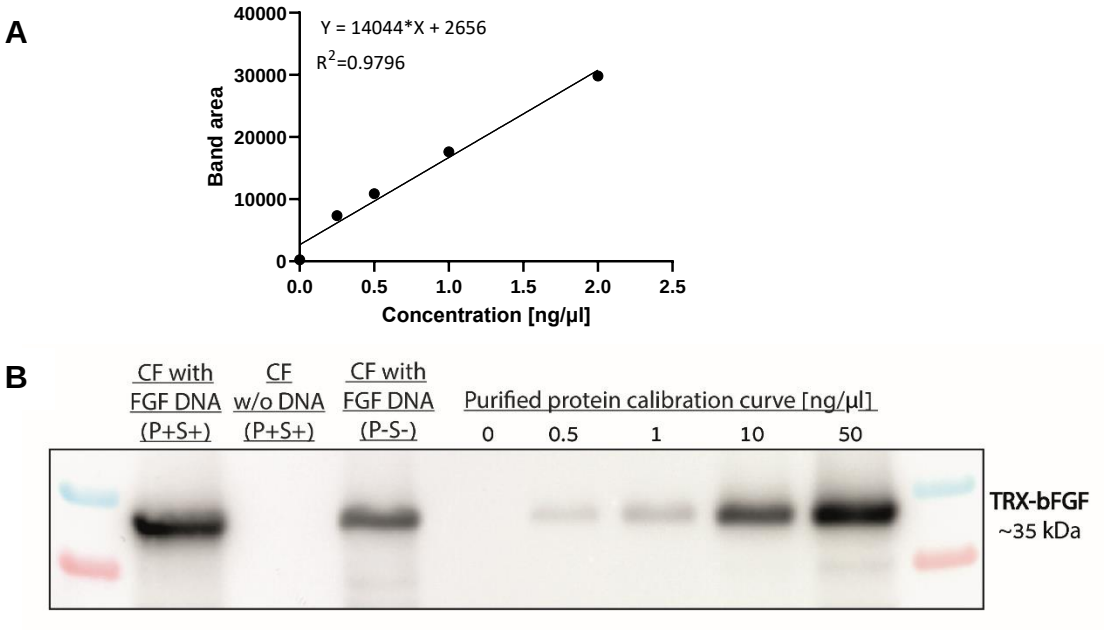


Figure S5. TRX-FGF concentration analysis in SCs and cell-free bulk reaction. (A) A representative calibration curve of band area quantified using ImageJ gel analysis plugin, versus purified TRX-FGF concentrations [ng/µl] and used to calculate the produced TRX-FGF concentration in SCs. **(B)** western blot analysis of TRX-FGF produced in a cell-free bulk reaction, (P=PEG, S=Sucrose that were included/not included (+/-) in the reaction mixture). For reaction with PEG and sucrose, the evaluated FGF concentration is 135 $[\frac{\mu g}{ml}]$, (n=1).

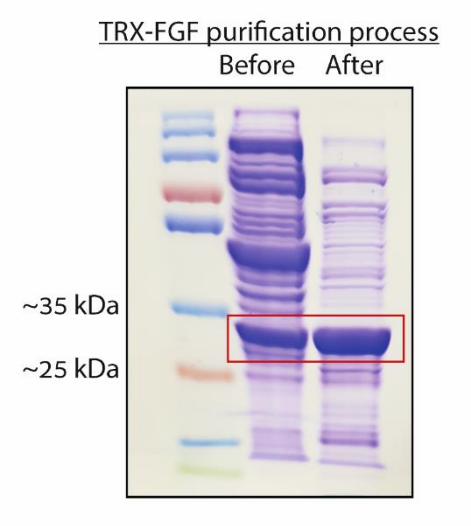


Figure S6. TRX-FGF purification process. SDS-PAGE gel stained with Coomassie blue of fractions taken before and after TRX-FGF purification process. PageRuler plus prestained protein ladder (Cat. 26619, Thermo Scientific™) was used as a marker. TRX-FGF (~35 kDa) protein is marked in the red square.

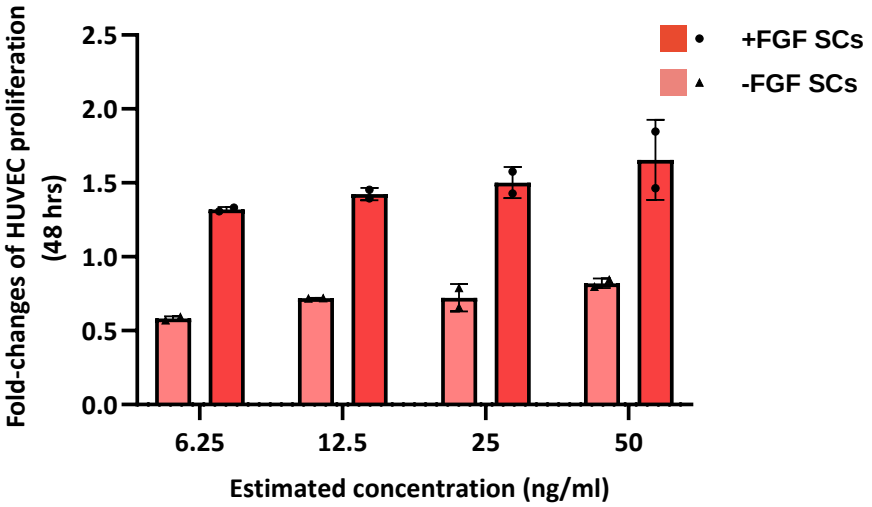


Figure S7. Determination of SCs treatment dose for *in-vitro* setups. HUVECs were treated with SCs with/without FGF DNA template in serially diluted concentrations to determine optimal treatment concentration for in-vitro experiments. Gradually increased cell proliferation was detected as SCs concentration was higher (6.25-50ng/ml estimated concentrations, according to the western blot concentration analysis). Data is presented as mean±SD (n=2); n represents independent samples in each group.

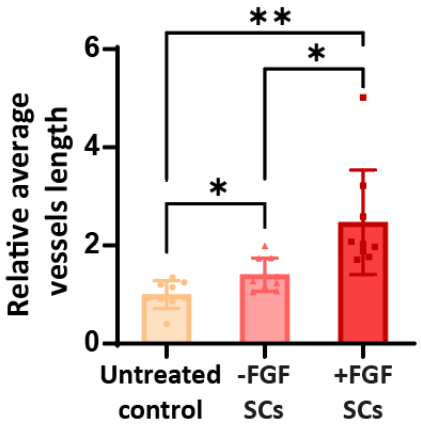


Figure S8. Computerized image analysis presents a significantly higher average vessel length in +FGF SCs treatment than -FGF SCs and untreated control. Values were normalized to the untreated control values. The analysis includes a value of 5.018, +FGF SCs, which was detected as an outlier by the ROUT (Q=1%) test. (n=9); *P<0.0468, **P<0.0087.

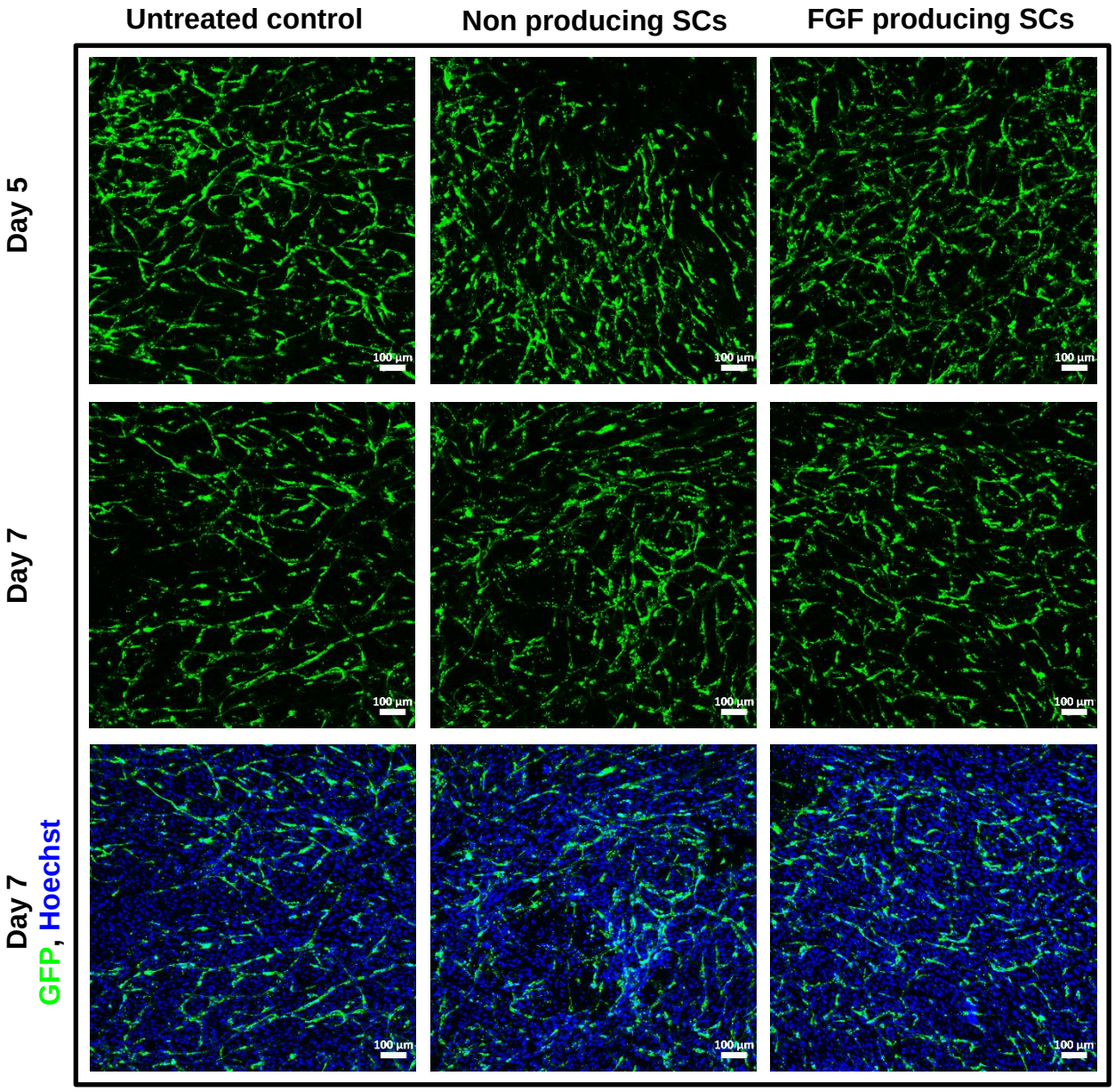


Figure S9. Effect of single treatment on vascular self-assembly in engineered scaffolds. GFP-expressing HUVECs (green) and DPSCs were co-seeded on CelGro constructs. Single treatments of +/- FGF SCs or without treatment were applied on the day of cell seeding. A slight vascular formation was observed five days post-seeding. Vessel destabilization and rupture were seen on day seven post-seeding and reduced HUVECs viability. Cell nuclei were stained on day 7 using Hoechst (blue) to spot the DPSCs.

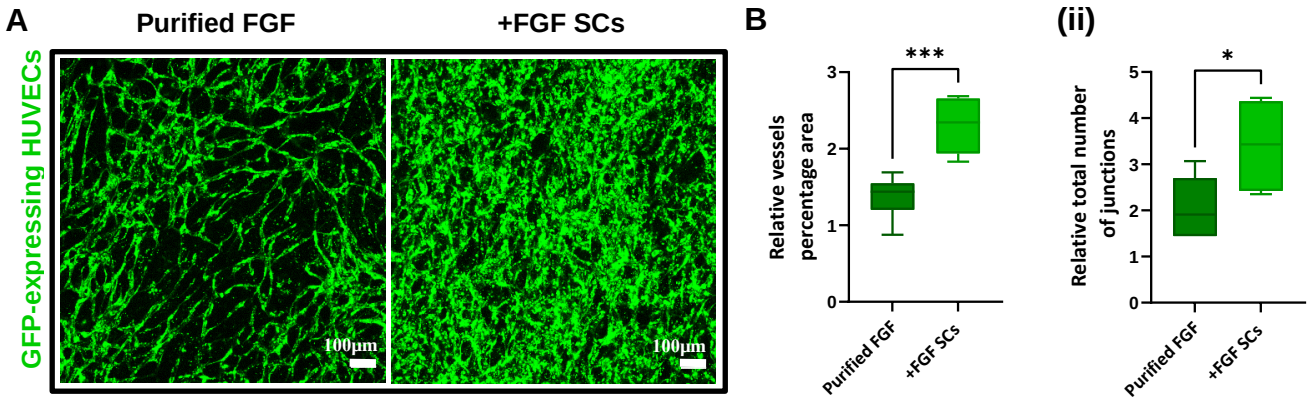


Figure S10. Vascular network formation after purified FGF and FGF synthetic cell treatments. (A) Confocal maximum intensity projection images of vascular network formation, induced by treatment of either purified TRX-FGF (50ng/ml) or FGF-producing SCs (production was estimated as 50ng/ml). **(B) (i)** Vessels % area and the **(ii)** total number of junctions quantified in the obtained vessel networks. Both analyses show significantly higher values after treatment with FGF-producing SCs. Values were normalized to untreated cell control. Data is expressed as a mean ± SD (n=6 independent samples); Unpaired t-test P-value; *P=0.0239, ***P=0.0008.

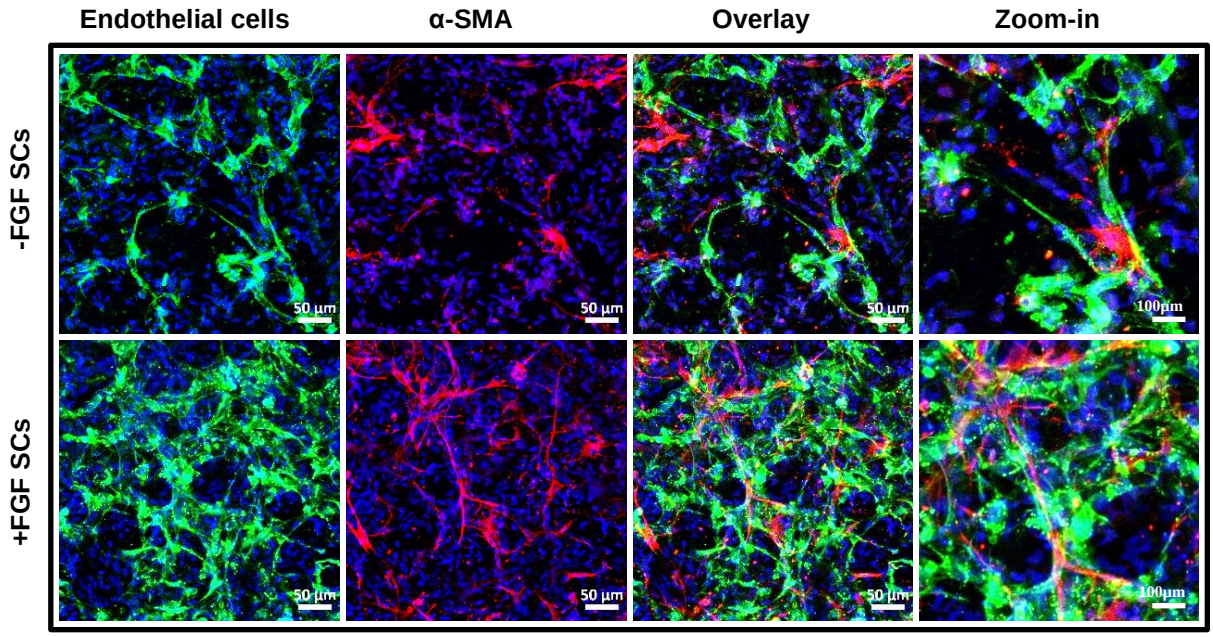


Figure S11. Expression of αSMA in supporting cells and proximity to GFP-expressing vessels. Maximum intensity projection images of scaffolds fixed after seven days from seeding and stained for VE-Cadherin (green), a-SMA (red), and cell nuclei (DAPI, blue). Co-localization of elongated a-SMA cells with ECs is shown when treated with FGF-producing SCs.

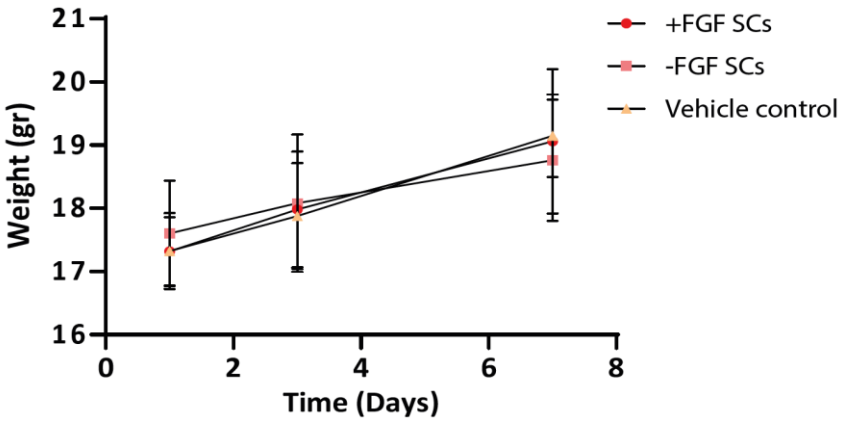


Figure S12. SC treatments do not affect mice's body weight. Mice were weighed on days 1,3 and 7 of the Matrigel plug experiment. Gradually body weight increase was measured for all participating animals. n=5 mice for each treatment. Data represent mean±SD.

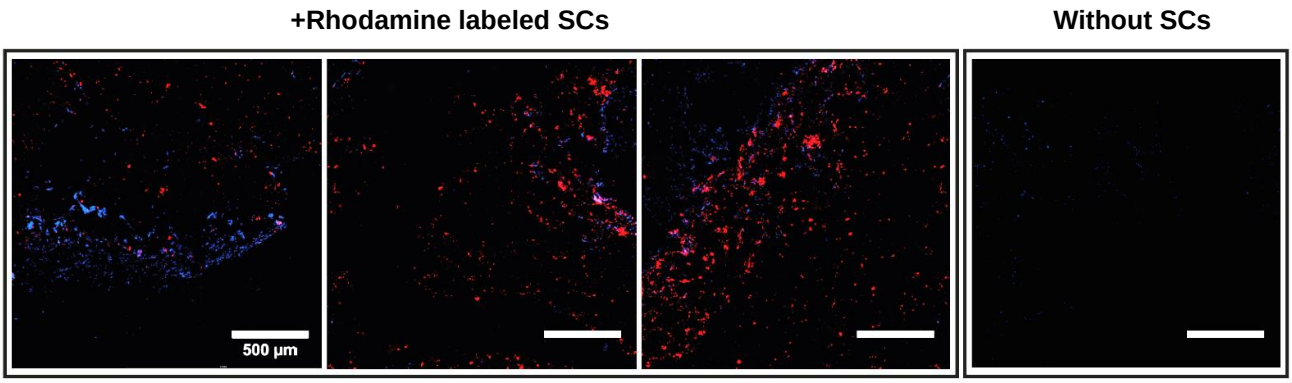


Figure S13. SCs in Matrigel plug. Representative images of Matrigel plug section with rhodamine-labeled SCs (red). Host cells were contour stained with DAPI (blue). Mice were subcutaneously injected with rhodamine-labeled SCs-enriched Matrigel and sacrificed 24hrs later. Plug without SCs served as control. Scale bar=500µm.

Table S1. Used phospholipids and their hydrocarbon chain characteristics.

Phospholipid full name	Abbreviation	Fatty acid length and saturation
1,2-dioleoyl-sn-glycero-3-phosphocholine	DOPC	18:1
L-α-phosphatidylcholine, hydrogenated (Soy)	HSPC	16:0 (11.4%), 18:0 (88.6%)
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine	POPC	16:0-18:1
L-α-phosphatidylcholine	Egg-PC (EPC)	**
1,2-dipalmitoyl-sn-glycero-3-phosphocholine	DPPC	16:0
1,2-dimyristoyl-sn-glycero-3-phosphocholine	DMPC	14:0

** A naturally derived phospholipid mixture from egg-yolk, mainly 16:0-18:1.

Table S2. Scanned lipid compositions and their suitability for the construction of GUVs in the emulsion transfer method.

Lipid composition (Weight ratios)	**Suitability for GUVs preparation using w/o method**
HSPC:Cholesterol (1:1)	No
POPC:HSPC:Cholesterol (4:3:1)	No
DOPC: Cholesterol (1:1)	No
POPC: DOPC: Cholesterol (4:3:1)	**Yes**
POPC: Cholesterol (1:1)	**Yes**
Egg-PC: Cholesterol (1:1)	**Yes**
DPPC: Cholesterol (1:1)	No
POPC:DPPC:Cholesterol (4:3:1)	No
DMPC: Cholesterol (1:1)	No
POPC:DMPC: Cholesterol (4:3:1)	No

Table S3. Protein sequences.

Name	Sequence
TRX-6His-hbFGF (Mut)	ATGAGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAG CGGACGGGGCGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATG ATCGCCCCGATTCTGGATGAAATCGCTGACGAATATCAGGGCAAACTGACCGTTGCA AAACTGAACATCGATCAAAACCCTGGCACTGCGCCGAAATATGGCATCCGTGGTATC CCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCAAAGTGGGTGCACT GTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCCCACCATCACCACCA CCACGACGACGATGATAAAGCTGCAGGTTCTATCACCACCCTGCCGGCTCTGCCGG AAGACGGTGGTTCTGGTGCGTTCCCGCCGGGTCACTTCAAAGATCCGAAACGTCTGT ACTGTAAAAACGGTGGTTTCTTCCTGCGTATCCACCCGGATGGTCGTGTTGATGGTG TTCGTGAAAAATCTGATCCGCACATCAAACTGCAGCTGCAGGCTGAAGAACGTGGTG TTGTTTCTATCAAAGGTGTTTCTGCTAACCGTTACCTGGCTATGAAAGAAGACGGTCG TCTGCTGGCTTCTAAATCTGTTACCGATGAATGTTTCTTCTTCGAACGTCTGGAATCTA ACAACTACAACACCTACCGTTCTCGTAAATACACCTCTTGGTACGTTGCTCTGAAACG TACCGGTCAGTACAAACTGGGTTCTAAAACCGGTCCGGGTCAGAAAGCGATCCTGTT CCTGCCGATGTCTGCTAAATCTTGA
sf-GFP-6His	CATATGAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAG ATGGTGATGTTAATGGGCACAAATTTTCTGTCCGTGGAGAGGGTGAAGGTGATGCTA CAAACGGAAAACTCACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATG GCCAACACTTGTCACTACTCTGACCTATGGTGTTCAATGCTTTTCCCGTTATCCGGAT CACATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAA CGCACTATATCTTTCAAAGATGACGGGACCTACAAGACGCGTGCTGAAGTCAAGTTT GAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATG GAAACATTCTCGGACACAAACTCGAGTACAACTTTAACTCACACAATGTATACATCAC GGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGCCACAACGTTGAA GATGGTTCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCC CTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGTCCTTTCGAAAGATCC CAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTAC ACATGGCATGGATGAGCTCTACAAAGGAGGGTCCCATCACCATCACCATCACTAAGG ATCC

Table S4. Primers for plasmid design.

Primer	Sequence
TRX-6His-hbFGF forward (NcoI)	TGTACCCATGGATGAGCGATAAAATTATTCACC
TRX-6His-hbFGF reverse (XhoI)	TCGCTCGAGTCAAGATTTAGCAGACATCG

Table S5. Synthetic cells' inner reaction composition based on S30-T7 lysate and its corresponding outer solution.

Reagent	Inner solution – final concentration	Outer solution – final concentration
HEPES KOH (pH=8)	55mM	83 mM
Magnesium acetate	14mM	21 mM
Potassium acetate	50mM	76 mM
Ammonium acetate	155mM	236.4 mM
Polyethylene glycol 6000 (PEG)	3% (w/v)	4.5% (w/v)
3-Phosphoglyceric acid (3-PGA)	40mM	61 mM
Amino acids - mixture I	2.5mM	3.8 mM
Amino acids - mixture II	2.5mM	3.8 mM
ATP	1.2mM	1.8 mM
GTP	1mM	1.5 mM
UTP	0.8mM	1.2 mM
IPTG	1mM	1.5mM
Sucrose	200mM	-
Glucose	-	303mM
S30-T7 lysate	34% (v/v)	-
DNA.	10 ng/µl	-
Ultrapure water	To total reaction volume	18.3% (v/v)

Table S6. Synthetic cells' outer solution composition that was used with the PURE-based inner solution.

Reagent	Final concentration
HEPES KOH pH=8	83 mM
Magnesium acetate	21 mM
Potassium (K) acetate	76 mM
Ammonium acetate	236.4 mM
3-Phosphoglyceric acid (3-PGA)	61 mM
Amino acids – mixture I	3.8 mM
Amino acids – mixture II	3.8 mM
ATP	1.8 mM
GTP	1.5 mM
UTP	1.2 mM
H_2O UPW	44% (v/v)

Movie S1 (separate file). Time-lapse video of capillary-like tube formation of GFP-expressing HUVEC

GFP-labled HUVEC were grown on growth-factors reduced basement membrane matrix in the presence of +/- FGF SCs (left/right, respectively). Cellular tube formation was imaged in one-hour gaps over 18 hours using the Lionheart FX automated microscope.

Movie S2 (separate file). 3D confocal visualization of a highly vascular network formation, promoted by +FGF SCs. HUVEC-GFP and DPSCs on Celgro scaffold after one week of +FGF SCs' treatment. Endothelial cells are labled in green (anti-VE-Cadherin, GFP), Collagen-IV is marked in red (anti-Collagen-IV), cells nuclei are stained with DAPI (blue).

Practical volume (PV) = $Sum[\frac{4\pi\left(\frac{D}{2}\right)^3}{3}]$ [ml], D= SC diameter

Total tested volume (TV) = 0.0001271 [ml]

Evaluated FGF concentration in cell-free bulk reaction = 135 $[\frac{\mu g}{ml}]$

Or

Evaluated FGF concentration in SCs = 2.15±0.8 $[\frac{\mu g}{ml}]$

FGF concentration in practical volume $[\frac{\mu g}{ml}] = \frac{FGF\ Concentration\ [\frac{\mu g}{ml}]}{\left(\frac{PV}{TV}\right)[\frac{ml}{ml}]}$

Equation S1. FGF concentration in SCs practical reaction volume $[\frac{\mu g}{ml}]$. PV and TV parameters were obtained from the imaging flow cytometry of size analysis. FGF concentration was evaluated based on western blot analysis.

SI References

1. Adir O*, et al.* (2020) Preparing Protein Producing Synthetic Cells using Cell Free Bacterial Extracts, Liposomes and Emulsion Transfer. *Journal of Visualized Experiments* (158).
2. Krinsky N*, et al.* (2018) Synthetic Cells Synthesize Therapeutic Proteins inside Tumors. *Adv Healthc Mater* 7(9):e1701163.
3. Kaduri M*, et al.* (2021) Targeting neurons in the tumor microenvironment with bupivacaine nanoparticles reduces breast cancer progression and metastases. *Science Advances* 7(41).