Spheroid-based human endothelial cell microvessel formation *in vivo*

Anna M Laib[1,5], Arne Bartol[1,5], Abdullah Alajati[2,5], Thomas Korff[3], Holger Weber[4] & Hellmut G Augustin[1]

[1]Joint Research Division Vascular Biology of the Medical Faculty Mannheim (CBTM), University of Heidelberg, and the German Cancer Research Center Heidelberg (DKFZ), Im Neuenheimer Feld, Heidelberg, Germany. [2]Friedrich-Miescher-Institute for Biomedical Research, Maulbeerstrasse, Basel, Switzerland. [3]Department of Physiology and Pathophysiology, University of Heidelberg, Im Neuenheimer Feld, Heidelberg, Germany. [4]ProQinase GmbH, Breisacher Straße, Freiburg, Germany. [5]These authors contributed equally to this study. Correspondence should be addressed to H.G.A. (augustin@angiogenese.de).

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The study of angiogenic endothelial cells (ECs) has in recent years greatly stimulated multiple fields of vascular biology research. A number of cellular models and numerous complex developmental, manipulatory and tumor animal models have been developed to study angiogenesis *in vitro* and *in vivo*. To connect the versatility of cellular assays with the complexity of readouts of *in vivo* experimentation, we have developed an endothelial transplantation assay. This assay is based on grafting *ex vivo* generated EC spheroids (2 d) in a suitable matrix in immunocompromised mice, to give rise to a 3D network of capillaries (20 d). This vasculature connects to the mouse vasculature, is perfused and matures by recruiting mouse mural cells. Here we describe the detailed protocol for this assay, including generation of spheroids, injection into mice, excision and processing of resulting plugs, and quantification by immunohistochemical analysis of the resulting vasculature.

INTRODUCTION

Neovascularization occurs by mechanisms of vasculogenesis and angiogenesis. Vasculogenesis denotes the *de novo* formation of blood vessels during embryogenesis with progenitor cells migrating to sites of vascularization, differentiating into endothelial cells (ECs) and organizing the primary capillary plexus[1]. The budding of new capillary branches from pre-existing blood vessels is referred to as angiogenesis, and it involves complex interactions between vascular cells and the corresponding extracellular environment[2]. Numerous angiogenic factors, such as the vascular endothelial growth factors (VEGF), the fibroblast growth factors (FGF) and the angiopoietins activate ECs, which in turn proliferate and migrate to form a lumenized three-dimensional capillary network[3]. Guidance molecules from the Eph/ephrin, Robo/Slit, Unc/Netrin and Neuropilin/Plexin/Semaphorin receptor/ligand families exert attractive and repulsive signals, thereby orchestrating the vascular assembly process[4,5]. Eventually, the assembled tubular system is covered by pericytes and SMCs, which stabilize the resulting network and control the quiescent phenotype of the vascular endothelium[6].

The mechanistic understanding of individual steps of the angiogenic cascade has laid the rational basis for the development of angio-inhibitory therapies that are now clinically approved in the fields of oncology and ophthalmology[7]. Yet, the long-term effect of angiomanipulatory therapies (i.e., angio-inhibitory and angio-stimulatory) will likely exceed far beyond the limited clinical applications achieved thus far to include apparently unrelated disorders, such as peripheral and coronary vascular disease, wound healing as well as skin and joint disorders[8].

Robust and reliable experimental models are key to further advance the fields of basic vascular biology research, as well as the translational exploitation of such basic science knowledge. Numerous cellular and animal models have been developed towards this end[9]. These include cellular assays (e.g., migration, proliferation and capillary-tube formation) as well as *in vivo* models (e.g., tumor models, retina, corneal assay)[10–14]. To bridge the inherently reductionist approach of cellular assays with the complexity of *in vivo* experimentation, we have recently established an EC spheroid-based grafting assay that allows the engineering of a complex three-dimensional capillary network from transplanted human ECs[15]. This assay has a remarkable efficacy (capillary network formation from less than 10^5 cells), flexibility (can be adapted for multiple applications) and robustness (quantitative analysis of all experimental parameters). The principle of this assay is based on the sprouting and network formation originating from multiple, gel-embedded aggregated ECs (spheroids of 500–1,000 cells). As such, grafted EC spheroids serve as multiple starting points for the sprouting of EC, which most closely mimics the behavior of ECs during sprouting angiogenesis. Over a period of 20 d, such grafted EC spheroids give rise to a complex network of capillaries that anastomoses with the mouse vasculature to yield a fully functional and perfused vascular network[15].

Here, we describe in detail our most up-to-date procedure for the establishment of a simple and robust spheroid-based EC implantation assay that can be exploited for numerous angiogenesis- and lymphangiogenesis-related applications. We have developed the EC spheroid implantation technique as an experimental model to study vessel formation originating from gain-of-function or loss-of-function manipulated ECs to identify novel candidate molecules of the angiogenic cascade. Similarly, we have established lentivirally transduced human umbilical vein endothelial cells (HUVECs) to over-express or down-regulate genes via shRNA for longer periods of time, to study manipulated primary human ECs in the assay. Similarly, the assay can be used for drug testing as well as for tissue engineering purposes[15]. Intra-peritoneal or oral applications work well in such drug testing studies. In contrast, we discourage repeated injections directly into the plug because of damaging the matrigel structure, and therefore the human vasculature. Likewise, the assay can be used for co-implantation experiments of ECs and tumor cells (TCs)[15]. The angiogenesis assay is additionally useful to investigate the potential of different

endothelial progenitor cells (EPCs) to generate human vessels *in vivo*. We recommend for such studies the solid characterization of the cells *in vitro* before injection to achieve the best possible experimental results.

Overview of the procedure

A general outline of the procedure and the duration for all individual steps are shown as a flow diagram in **Figure 1**. Spheroid production is started a day before injecting the mice. To assure a comparable size of the spheroids and to facilitate the production of large numbers of spheroids, one should use a multi-micropipette (**Fig. 2**). Before harvesting the spheroids on the second day (the day of injection), the adequate formation of uniformly rounded and sized spheroids can be observed under the microscope (**Fig. 2**). Subsequently, immunodeficient mice are subcutaneously injected with spheroids under sterile conditions. The matrigel/fibrin plugs are maintained in mice for 20–60 d, followed by the functional, biochemical and/or immunofluorescence evaluation, as outlined below. One of the most critical steps in the *in vivo* angiogenesis assay is the dissection of the matrigel/fibrin plugs. For investigators with little experience in animal experiments and matrigel based methods, it may be challenging to distinguish the implant from the mouse fat tissue and to remove the implant properly. We have addressed this issue by illustrating the dissection of matrigel plugs in detail in **Figure 3**.

Advantages and disadvantages of the technique

The key advantages of the spheroid implantation assay compared with other *in vivo* angiogenesis assays are as follows:

- Universal and versatile experimental protocol for the growth of human blood vessels *in vivo*, enabling numerous applications in the fields of vascular research and regenerative medicine[15], including usage of different genetically modified ECs, EPCs and ECs/TCs, and testing of different pro- and anti-angiogenic cytokines.
- Spheroidal delivery of ECs facilitates the generation of a complex vascular network from as few as 100,000 cells.

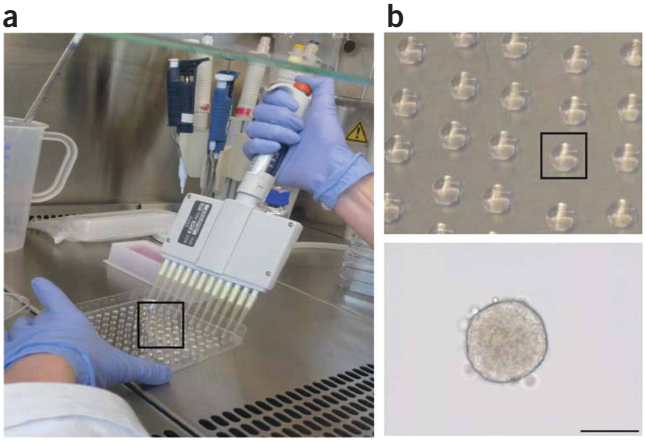

Figure 2 | Preparation of spheroids. (**a**) The usage of a multi-micropipette assures consistent and uniform 'hanging-drops' throughout a cell culture plate. (**b**) Magnification of a 'hanging-drop' containing a compact and round spheroid, which has been formed within 18 h. Scale bar, 100 µm.

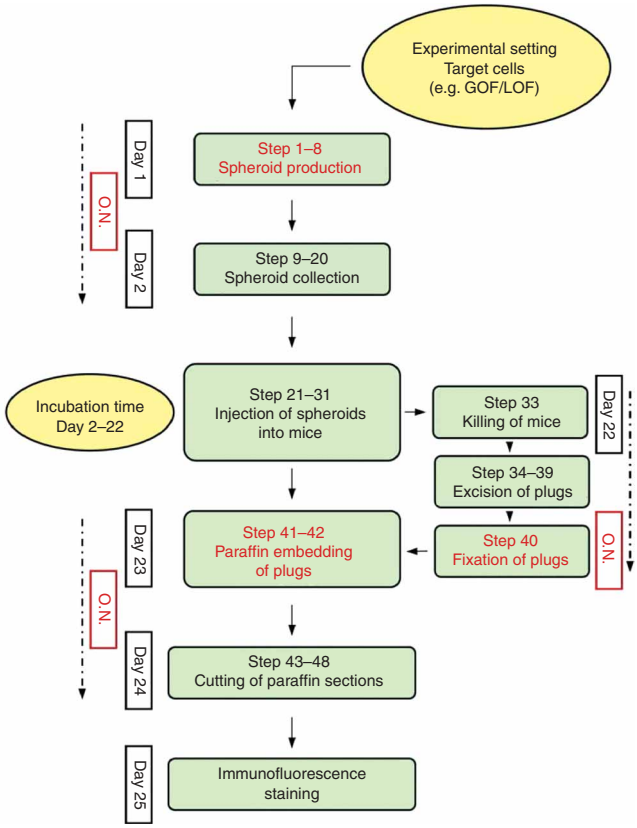

Figure 1 | Flow chart of the *in vivo* angiogenesis assay. Green squares indicate individual steps of the protocol. Expected time frames are indicated in black squares. Red squares indicate procedures that may be done overnight. Yellow ovals indicate alternative steps, which may be necessary depending on the specific experimental settings (GOF, gain-of-function; LOF, loss-of-function; O.N., overnight).

- The assay is highly effective using a mixture of matrigel and fibrin (1:1), a matrix that proved empirically superior to collagen, matrigel or fibrin matrices alone. Most notably, the addition of fibrin to matrigel circumvents the necessity of adding heparin for the retention of growth factors, and takes advantage of the release kinetics and longer-lasting effects of fibrin-immobilized angiogenic growth factors[16].
- As a single cell type-based assay, the interpretation of experimental findings is more straightforward compared to complex multicellular animal models.
- Endothelial cells from any origin can be used for spheroid formation and injection, (e.g., human umbilical artery endothelial cells (HUAECs), human saphenous vein endothelial cells (HSVECs), human dermal microvascular endothelial cells (HDMECs) and human dermal lymphatic endothelial cells (HDLECs))[15].

The main disadvantages of this assay are the following:

- Matrigel is chemically undefined and shows variations between batches.
- The assay is versatile and standardized. Yet, there is considerable variation in the macroscopic 3D structure of the resulting plug despite a standardized injection volume. The reliable quantification of blood vessel parameters consequently needs an adequate number of experimental replicates. We recommend a number of at least 5–7 mice per group.

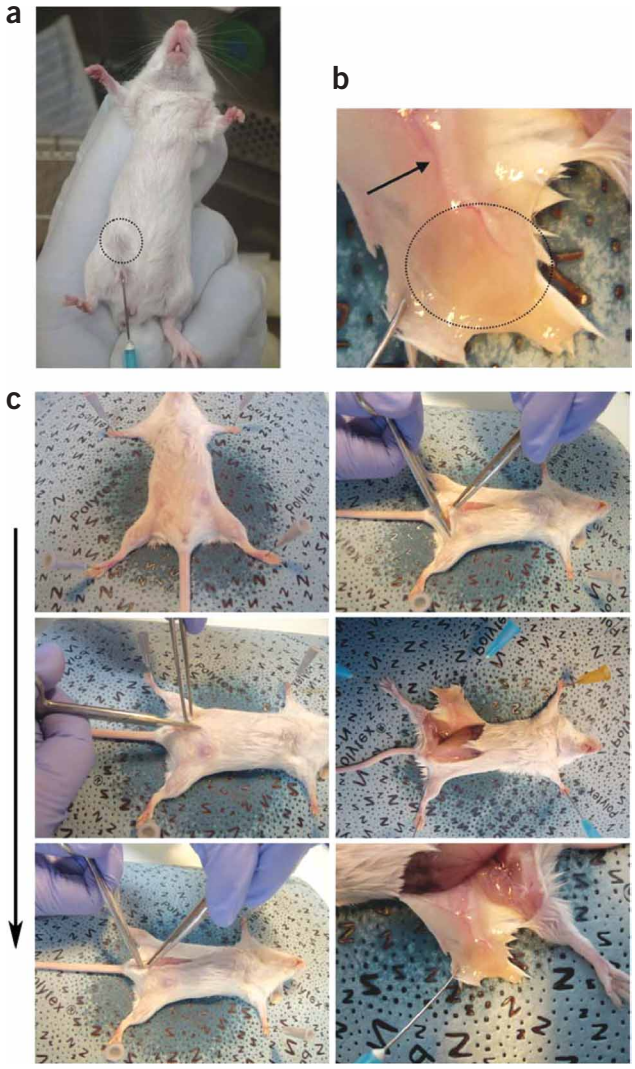

Figure 3 | Injection and dissection of a matrigel–fibrin plug. (**a**) Subcutaneous injection of the matrigel/methocel/fibrinogen/endothelial cell basal medium (ECBM) matrix. Inject the matrigel plugs subcutaneously into the abdominal flanks. If done correctly, a small bulge is detectable in the skin. (**b**) Magnification of a plug. The black circle indicates the matrigel–fibrin plug, whereas the black arrow indicates a mouse artery, which is surrounded by connective tissue. (**c**) (Pictures are arranged vertically, beginning with the upper left picture.) After sacrificing the mice, lay them on their backs. Stretch and pin limbs to ease dissection of the matrigel–fibrin plug (upper left). Moisten the abdomen with 70% ethanol to see plugs appear as bumps on the left and right side of the animal. Start to cut with a sharp pair of scissors along the abdominal midline. Originating from the longitudinal cut, carefully dissect the plug with some surrounding tissue for orientation when histology is carried out from the skin and remove it (lower right). All animal procedures were carried out in accordance with the guidelines outlined by the local and national committees for animal experiments.

- The use of matrigel and thrombin/fibrinogen may be accompanied by an inflammatory reaction in the surrounding tissues[17]. Immune cells and pro-inflammatory stimuli may contribute to the angiogenic response.
- The xenograft character of the assay needs the use of immunodeficient mice; the strain of mice used may be critical (see Experimental design).
- A common problem associated with various assays of vascularization into matrix implants is the nonspecific inflammatory host response to the implant. Therefore, caution needs to be exercised in the interpretation of experimental data and proper controls need to be analyzed (see below). The EC spheroid-grafting assay is not without an inflammatory component. Yet, this does not significantly interfere with the versatility of the assay to, e.g., qualitatively study the effect of different growth factors such as VEGF and FGF-2[15].

Experimental design

Preparation of spheroids. Endothelial cells are cultured in hanging drops of 25 µl medium containing methocel (20% methocel stock solution (for preparation of methocel see REAGENT SETUP) and 80% culture medium (supplements/fetal calf serum (FCS): cell type-dependent)) each to aggregate as 'spheroids'. For the preparation of spheroids, it is important to use viable cells at low passage number (passage 3–4). Before harvesting the spheroids, monitor if the cells have formed evenly rounded spheroids, by observing them under a low magnification microscope.

Cell-type dependent media and supplements. It is essential to use cell-type dependent media for the growth of cells and the preparation of spheroids. For example, HUVEC from PromoCell are cultured in endothelial cell growth medium (ECGM) with Supplement Mix (PromoCell, cat. no. C-22010) that is supplemented with 10% FCS (vol/vol) and 1% penicillin–streptomycin (vol/vol).

Thrombin. Thrombin is used for the generation of fibrin from fibrinogen. Fibrin binds growth factors, thereby releasing them over a long period of time. In addition, fibrin induces the expression of several pro-angiogenic genes.

Use of matrigel. Matrigel is a re-constituted basement membrane extract, which is rich in laminin, collagen IV and entactin. It is chemically undefined and shows variations between batches. Therefore, one should stock sufficient supplies to minimize lot-to-lot variations. Basement membrane can also be purified from Engelbreth–Holm–Swarm (EHS) sarcoma-derived extracellular matrix[18]. The EHS sarcoma is a rich source of individual basement-membrane components and matrigel. As we are only experienced in the use of matrigel from BD, we cannot comment on results with other basement membranes in our assay.

Fixation solutions. All described immunohistochemical staining procedures in this protocol were carried out with Roti–Histo-Fix 4% (4% paraformaldehyde). Proper fixation conditions need to be established individually for other staining protocols.

Controls. Unmodified HUVEC spheroids stimulated with VEGF-A and basic fibroblast growth factor (bFGF) (1,000 ng ml^{-1}) were routinely used as experimental controls. Appropriate experiment-specific controls need to be included, as needed.

Mouse strain. Female CB17 severe combined immunodeficiency (SCID) mice (Charles River, Sulzfeld), 6–8 weeks of age. Mice should be kept under specific pathogen-free conditions. All animal

procedures have to be carried out in accordance with the guidelines outlined by the local committee for animal experiments. It is important to use CB17 SCID mice as they lack B and T cells, whereas Nude mice only lack T cells. We can only comment on the use of SCID mice, as we only have preliminary experience with the use of Nude mice.

Angiogenic stimulants. Activities of several different angiogenic factors can be tested in the assay. We have shown that VEGF in combination with FGF-2 are potently angiogenic in the assay[15]. When individually applied, both factors proved to induce the sprouting of capillaries. Although FGF-2 stimulation led to the formation of a perfused, mural cell-covered neovascularization, VEGF stimulation led to a comparable number of newly formed vessels, but these were less perfused and covered by mural cells. Co-stimulation of VEGF and platelet derived growth factor BB (PDGF-BB), as well as co-implantation of human umbilical artery smooth muscle cells (SMCs) or normal human dermal fibroblasts proved to be potent settings for the assay[15].

MATERIALS

REAGENTS

- Basic fibroblast growth factor (FGF-2, RELIATech, cat. no. 300-003L) ▲ CRITICAL If a large number of experiments are anticipated, it is recommended that a large batch be obtained, aliquoted, tested and stored at −80 °C.
- Bovine serum albumin (BSA, type H1, GERBU, cat. No. 1063)
- Calcium acetate hydrate (Sigma-Aldrich, cat. no. 62-54-4) ! CAUTION Irritant, avoid contact to eyes, respiratory system and skin.
- Citrate buffer 10×, pH 6 ready to use (Dako, cat. no. S2369)
- Diaminobenzidine (DAB) substrate chromogen system (Dako, cat. no. K3466) ! CAUTION Irritant, limited evidence of a carcinogenic effect, avoid contact to eyes and skin.
- Dako Pen (Dako, cat. no. S2002) ! CAUTION Irritant, avoid contact with skin, harmful by inhalation.
- Dimethyl sulfoxide (DMSO, Carl Roth, cat. no. 4720.1)
- Donkey anti-rabbit, Alexa Fluor 546 (Invitrogen, cat. no. A11071)
- Donkey serum (Sigma-Aldrich, cat. no. D9663)
- Dulbecco's phosphate buffered saline without calcium and magnesium (PBS, PAA Laboratories, cat. no. H15-002)
- Endothelial cell basal medium (ECBM, PromoCell, cat. no. C-22210) ▲ CRITICAL To guarantee the optimal performance, cell-specific media should be used.
- Endothelial cell growth medium with SupplementMix (ECGM, PromoCell, cat. no. C-22010) ▲ CRITICAL To guarantee the optimal performance, cell-specific media should be used.
- Ethanol (Riedel-de Haën, cat. no. 32205)
- Fetal calf serum (FCS, PAA Laboratories, cat. no. A15-T04)
- Fibrinogen, human plasma (Calbiochem, cat. no. 341576)
- Fluoresceinisothiocyanate (FITC)-Dextran (70,000 MW, 25 mg/ml anionic, lysine fixable) (Invitrogen, cat. no. D1822)
- Fluorescent mounting medium (Dako, cat. no. S3023)
- Goat anti-mouse, Alexa Fluor 488 (Invitrogen, cat. no. A11001)
- Goat anti-mouse, Alexa Fluor 546 (Invitrogen, cat. no. A11003)
- Goat anti-rat, Alexa Fluor 488 (Invitrogen, cat. no. A11006)
- Goat serum, ready to use (Zymed, cat. no. 50062Z) ! CAUTION Harmful, avoid contact with skin.
- Hoechst dye 33258 (Sigma-Aldrich, cat. no. B2883-100MG)
- Isopropanol (VWR, cat. no. 1.09634.2511)
- KCl (Carl Roth, cat. no. 6781.1)
- KH_2PO_4 (Carl Roth, cat. no. 3904.1)
- Lectin, *Bandeiraea simplicifolia*, tetramethyl rhodamine isothiocyanate (TRITC) conjugated (Sigma, cat. no. L5264)
- Matrigel, growth factor reduced (BD Biosciences, cat. no. 354230)
- Methanol (Riedel-de Haën, cat. no. 65543)
- Methyl cellulose (Sigma-Aldrich, cat. no. M0512)
- Meyer's Hemalaun (Carl Roth, cat. no. T865.3)
- Mouse anti-human α-smooth muscle actin (SMA)-Cy3, monoclonal, 1A4, cat. no. C6198) ▲ CRITICAL As the described staining procedure has been specifically established, usage of this specific antibody is recommended.
- Mouse anti-human CD31, monoclonal, JC70/A (Dako, cat. no. M0823) ▲ CRITICAL As the described staining procedure has been specifically established, usage of this specific antibody is recommended.
- Mouse anti-human CD34, monoclonal, QBEND10 (Menarini, cat. no. 36686) ▲ CRITICAL As the described staining procedure has been specifically established, usage of this specific antibody is recommended.
- NaCl (Riedel-de Haën, cat. no. 7647-14-5)
- NaH_2PO_4 (Neolab, cat. no. 4770)
- Paraffin, solidification point 56–58 °C (Merck, cat. no. 8002742)
- Pertex (Medite, cat. no. 41-4010-00)
- Penicillin–streptomycin, 100×, 10,000 U/10 mg ml^{-1} (PAA Laboratories, cat. no. P11-010)
- Proteinase K (Sigma-Aldrich, cat. no. P2308-25MG)
- Rabbit serum (Sigma-Aldrich, cat. no. R4505-100ML)
- Rat anti-mouse CD31, monoclonal, MEC 13, 3 (BD Pharmingen, cat. no. 557355) ▲ CRITICAL As the described staining procedure has been specifically established, usage of this specific antibody is recommended.
- Roti–Histo-Fix 4% (pH 7) (Carl Roth, cat. no. P087.3) ! CAUTION Toxic, avoid contact to skin, eyes and mucous membrane, use in a chemical hood.
- Roti liquid barrier marker (Carl Roth, cat. no. AN92.1) ! CAUTION Harmful, avoid inhalation and contact to the skin.
- Target retrieval solution, citrate pH 6 (Dako, cat. no. S2369)
- Thrombin, human plasma (Calbiochem, cat. no. 605190)
- Trypsin-EDTA solution (10×) (PAA, cat. no. L11-003)
- Tween20 (Gerbu, cat. no. 2001)
- VEGF-A (165) (recombinant human, RELIATech, cat. no. 300-036) ▲ CRITICAL If a large number of experiments are anticipated, it is recommended that a large batch be obtained, aliquoted, tested and stored at −80 °C.
- Xylol (Merck, cat. no. 108681) ! CAUTION Harmful, avoid inhalation, contact to skin, eyes and mucous membrane, use in a chemical hood.
- Zinc acetate (Sigma-Aldrich, cat. no. 383317) ! CAUTION Mildly toxic, harmful when swallowed and avoid contact to eyes.
- Zinc chloride (Riedel-de Haën, cat. No. 14422) ! CAUTION Harmful when swallowed, avoid contact to eyes and skin.

Cells and media

- Human umbilical vein endothelial cells (HUVECs, pooled) (PromoCell, cat. no. C-12203) ▲ CRITICAL It is important to use pooled primary HUVECs to avoid donor specific responses. We observed that HUVECs from PromoCell are more viable and robust compared with other commercially available HUVECs.

Animals

- Female CB17 SCID mice (Charles River), 6–8 weeks of age ! CAUTION Mice should be kept under specific pathogen-free conditions. All animal procedures have to be carried out in accordance with the guidelines outlined by the local and national committees for animal experiments.

EQUIPMENT

- 1.5 ml and 2 ml reaction tubes
- 15 ml and 50 ml reaction tubes
- Centrifuge (IEC MultiRF, Thermo Electron Corporation, cat. no. 3590F)
- Clean bench (HeraSafe, Thermo Electron Corporation, cat. no. 1828)
- CO_2 gas incubator (HeraCell240, Thermo Electron Corporation, cat. no. 51019560)
- Dissecting instruments (Carl Roth, cat. no. 0829.1)
- Embedding cassette (Medim Histotechnologie, cat. no. 10-0114)
- Embedding machine (STP 120, Microm, cat. no. 40257535)
- Injection needle 0.6 × 30 mm, 23G 1/4″–Nr.14 (BD, cat. no. 300700)
- Inverted fluorescence microscope (IX81, Olympus, cat. no. E0432000)

TABLE 1 | Reagent Setup.

Solution	Components	Volume	Amount	Final concentration	Storage conditions
Methocel	Carboxymethylcellulose	—	6 g	1.2% (wt/vol)	Storable at 4 °C for at least 3 months
	ECBM	500 ml	—		
PBS (10×)	NaCl	—	400 g	1.34 M	Storable indefinitely at RT
	KCl	—	10 g	27 mM	
	Na_2HPO_4	—	57.5 g	200 mM	
	KH_2PO_4 ph 7.4	—	10 g	4.7 mM	
	H_2O	5 l	—		
PBST (10×)	NaCl	—	400 g	1.34 mM	Storable indefinitely at RT
	KCl	—	10 g	27 mM	
	Na_2HPO_4	—	57.5 g	200 mM	
	KH_2PO_4 ph 7.4	—	10 g	4.7 mM	
	Tween 20	5 ml	—	0.1% (vol/vol)	
	H_2O	5 l	—		
TBST (10×)	5 M NaCl	200 ml	—		Storable indefinitely at RT
	1 M TRIS pH 7.4	100 ml	—		
	Tween 20	10 ml	—	0.1% (vol/vol)	
	H_2O	Up to 10 l	—		
Zinc fixative	0.1 M TRIS, pH 7.4	1 l	—	—	Prepare fresh for each experiment
	Calcium acetate ($C_4H_6O_4Ca$)	—	0.5 g	3.2 mM	
	Zinc acetate $Zn(CH_3CO_2)_2$	—	5 g	27.3 mM	
	Zinc chloride ($ZnCl_2$)	—	5 g	36.7 mM	

ECBM, endothelial cell basal medium; PBS, phosphate buffered saline.

- Microcentrifuge with rotor for 2, 1.5 and 0.5 ml tubes (Biofuge pico, Heraeus, cat. no. 75003235)
- Microtome (HM 355S, Microm, cat no. 40257539)
- Microscope coverglasses (VWR International, cat. no. 631-0146)
- Microscope glass slides (Menzel-Gläser, cat. no. J1800AMNZ)
- Multi-micropipette (Starlab, cat. no. G9912-0200)
- Neubauer cell-counting chamber (Marienfeld, cat. no. 0640010)
- Plastic square petri dishes, 120/120/17 (Greiner bio-one, cat. no. 688102)
- Plastic cell culture flask (TPP, cat. no. 90026)
- Pipettes for volumes 500–1,000 µl
- Pipette tips for volumes 500–1,000 µl
- Section flattening plate (SW 85, Adamas Instrumenten bv, cat. no. 40257330)
- Standard plastic pipettes (2, 10 ml)
- Slide storage boxes (Carl Roth, cat. no. K532.1)
- Syringe (Dispomed, cat. no. 22001)
- Timer (Oregon Scientific, cat. no. TR 118)
- Water bath with accurate temperatures between 15 and 99 °C (e.g., TW 12, Julabo, cat. no. 9550112)

REAGENT SETUP

Self-made solutions

- PBS (see **Table 1**)
- PBST (phosphate buffered saline with tween) (see **Table 1**)
- TBST (tris-base buffered saline with tween) (see **Table 1**)
- Zinc-Fixative (see **Table 1**)

Methocel Autoclave carboxymethylcellulose (6 g) in a 500 ml glass bottle containing a carefully cleaned magnetic stir bar. Preheat 500 ml of ECBM in a 60 °C water bath. Add 250 ml of the preheated ECBM to the autoclaved carboxymethylcellulose and mix under sterile conditions for 20 min. Add 250 ml of ECBM to the solution and mix overnight at 4 °C. Divide the solution evenly into 50 ml tubes and centrifuge them for 3 h at 3,500*g* at 4 °C. Make sure that the tubes withstand the centrifugation step. The solution can be kept at 4 °C for ~3 months. ! **CAUTION** As the sediment contains residual cellulose fibres, only the upper 45 ml from the 50 ml aliquots should be used for the experiment.

Methocel/fibrinogen/ECBM mixture The mixture is prepared in a ratio of 1:1:1 (vol/vol/vol) (1 ml methocel + 1 ml fibrinogen + 1 ml ECBM per plug). Fibrinogen should be thawed at room temperature (RT, 20–24 °C), as it will precipitate if stored on ice. When preparing the mixture, fibrinogen and ECBM should be pipetted first. Cutting off the tip of the pipette with sterile scissors is recommended. This improves proper aspiration of the high-viscosity methocel solution.

Before the assay Prepare reagents and animals.

Order mice and matrigel.

- Thaw matrigel at 4 °C overnight or on ice on the day of injection.
- Prepare syringes and needles.
- Re-suspend angiogenic factors or inhibitors.

Before plug implantation Prepare the following equipment:

- Pipettes for ~1,000 µl and ~10 µl.
- Pipette tips.
- Ice bucket with matrigel and thrombin.
- Rack for reaction tubes.
- Syringes and needles.
- Tool for mouse earmarks.

PROCEDURE

Spheroid preparation for four mice ● TIMING ~3 h

1| This protocol is sufficient to generate 8 plugs of 1,000 spheroids each containing 100 ECs per spheroid. First, wash a confluent HUVEC monolayer with 1× PBS and aspirate the PBS.

2| Trypsinize the cells with 1× Trypsin-EDTA solution (diluted in PBS) for ~2 min (for a 10 cm^2 dish, you need 1 ml 1× Trypsin-EDTA solution).

3| Stop the reaction by pipetting 10 ml PBS (vol/vol) containing 10% FCS on the cells.

4| Pipette the cells into a reaction tube and sediment them by centrifugation (5 min at 150*g*, RT).

5| Discard the supernatant and resuspend the cells in a cell type-dependent media (in this case ECGM).

6| Count the cells and suspend 8×10^6 cells in 200 ml medium containing methocel (20% methocel stock solution and 80% culture medium (supplements/FCS: cell type-dependent), see REAGENT SETUP).

7| Divide the cell suspension evenly into non-adherent plastic square petri dishes using a 12-channel pipette (25 µl for each spheroid); for the generation of 1,000 spheroids (sufficient for one plug), ~5 plastic square petri dishes are needed.
▲ **CRITICAL STEP** Be aware that the right plates are used (non-adherent plastic square petri dishes).

8| Turn the plates upside down and incubate them in a humidified atmosphere at 37 °C (5% CO_2, for 24 h) (the cells in suspension will form hanging drops).
? TROUBLESHOOTING

Harvesting of spheroids ● **TIMING** ~**3 h**

9| Prepare 7 ml PBS containing 10% (vol/vol) FCS per plug (1,000 spheroids) (for four mice ~60 ml PBS/FCS is needed).

10| Pipette 5 ml of PBS/FCS on the first plastic square petri dish, containing the spheroids and spread over the plate by carefully turning the plate. Harvest the spheroids by removing the entire cell-containing medium using standard plastic pipettes (10 ml), and collect all cells from the same experimental group in labeled falcon tubes (50 ml reaction tubes). The mixture can be poured from one plate to another (around 4–5 times, ~4 dishes can be harvested with 5 ml PBS/FCS). Transfer the mixture of spheroids/PBS/FCS in a 50 ml tube when the fluid becomes too viscous.
▲ **CRITICAL STEP** Before harvesting the spheroids, check whether the cells have formed evenly rounded spheroids by observing them under a low magnification microscope.
▲ **CRITICAL STEP** Minimize the loss of spheroids by collecting the whole medium.
▲ **CRITICAL STEP** Avoid damaging the spheroids by gentle aspiration.

11| Sediment spheroids by centrifugation (5 min at 150*g*, without brake, RT).

12| Aspirate the supernatant.
▲ **CRITICAL STEP** Do not touch the spheroid pellet, and thereby aspirate the spheroids.

13| In the meantime, prepare 8 × 300 µl (+ 2 × 300 µl as safety margin) of methocel/fibrinogen/ECBM (1:1:1; see REAGENT SETUP) mixture for injecting four mice at RT.
▲ **CRITICAL STEP** As the methocel mixture is highly viscous, it is suggested to cut off the tip of a pipette by using sterile scissors, which will ensure accurate aspiration of the fluid.
▲ **CRITICAL STEP** Fibrinogen will precipitate when stored on ice.

14| Wash the spheroids once with 10 ml ECBM.

15| Sediment the spheroids by centrifugation (5 min at 150*g*, without brake, RT).

16| Carefully remove the supernatant.
▲ **CRITICAL STEP** If additional suspended cells should be added (e.g., fibroblasts, SMCs, pericytes, tumor cells…), add them to the reaction tube and centrifuge before distributing the spheroids in equal amounts to the reaction tubes.

17| Resuspend the spheroids in ECBM (volume: 1 ml per plug) and evenly divide the spheroids into 2 ml reaction tubes at RT (1 reaction tube per plug).

18| Collect spheroids by centrifugation (5 min at 1,300*g*, RT).
▲ **CRITICAL STEP** The spheroid sediment may be difficult to see and sometimes falls down from the edge of the tube to the bottom. Do not aspirate the spheroids.
? TROUBLESHOOTING

19| Add 1,000 ng ml^{-1} VEGF-A (165) and FGF-2 to the methocel/fibrinogen/ECBM mixture.
▲ **CRITICAL STEP** Thorough mixing of the growth factors in the methocel/fibrinogen/ECBM mixture is essential for reproducible assay results.
▲ **CRITICAL STEP** Avoid repeated freeze-thaw cycles of growth factors, as it may reduce their activity.

20| Add 300 µl of the growth factor supplemented methocel/fibrinogen/ECBM mixture to each spheroid sediment.

Plug implantation ● TIMING ~1 h

21| Earmark the mice.

! CAUTION All animal procedures have to be carried out in accordance with the guidelines outlined by the local committee for animal experiments.

22| Put the first reaction tube with the spheroids from Step 20 on ice.

▲ CRITICAL STEP The spheroid/methocel/fibrinogen/ECBM mixture must not be kept on ice for longer than half an hour. Otherwise the fibrinogen will polymerize and may not pass smoothly through the needle during injection.

23| Add 300 µl of matrigel on top of the spheroid/methocel/fibrinogen/ECBM mixture.

! CAUTION Remember to remove the matrigel from the freezer and store it on ice or at 4 °C in the refrigerator until it is thawed. It should be noted that the temperature of most refrigerators is usually adjusted to 8–10 °C, which may result in polymerization of the matrigel in some cases.

24| Add 4 µl (1 U μl^{-1}) of thrombin.

25| Carefully mix the solution by gently using a syringe (without a needle attached). Finally, aspirate the mixture with the syringe (avoid bubbles).

26| Attach a needle (0.6 × 30 mm, 23G 1/4" - Nr.14) to the syringe.

▲ CRITICAL STEP Injection must be carried out within 60–90 s, as the mixture solidifies quickly upon addition of thrombin. Therefore, we recommend using two people to inject; one should prepare the mixture, while the other one injects the mouse.

27| Remove all bubbles from the syringe.

28| Inject the mixture subcutaneously in the left or the right flank of the mouse as described in **Figure 3a**.

▲ CRITICAL STEP Make sure that the mixture is subcutaneously, not intraperitoneally, injected.

! CAUTION All animal procedures have to be carried out in accordance with the guidelines outlined by the local committee for animal experiments.

? TROUBLESHOOTING

29| After injection, wait for ~10 s to let the mixture solidify.

30| Repeat steps 22–29 with all samples.

31| To ensure the growth of a human capillary network, the animals should be kept under appropriately clean animal facility conditions for at least 20 d and ~60 d.

Dissection of implanted plugs ● TIMING ~1.5 h

32| Before excision, prepare the corresponding fixation solution (e.g., paraformaldehyde (PFA) and zinc-fixative; see REAGENT SETUP). For perfusion studies, inject 150 µl TRITC conjugated lectin in the tail vein of the mouse. Allow it to circulate for 15 min.

33| Kill the mouse by cervical dislocation.

▲ CRITICAL STEP The plugs must be immediately excised.

! CAUTION All animal procedures have to be carried out in accordance with the guidelines outlined by the local committee for animal experiments. In case of inexperience in surgical removal of organs or any other animal handling, the appropriate veterinarian should be contacted within the institution to request appropriate training.

34| Lay the mouse on its back. The abdomen should be moistened with 70% ethanol, which helps to locate the plug.

35| Stretch and pin the limbs to ease dissection.

36| Cut the skin open along the abdominal midline.

▲ CRITICAL STEP Do not penetrate the peritoneal cavity to assure correct removal of the skin from the peritoneum.

37| Originating from the longitudinal cut, dissect the skin on each flank as described in **Figure 3c**.

38| Carefully localize the border of the plug and dissect it very carefully from the skin to avoid damage.

? TROUBLESHOOTING

39| At this point plugs can be used for whole mount staining, as described in **Box 1,** or can be placed in an embedding cassette.

▲ CRITICAL STEP If different fixation conditions are to be tested, the plug may be cut into several pieces.

40| Transfer the cassettes to the corresponding fixation solution and incubate overnight at 4 °C.

BOX 1 | WHOLE MOUNT STAINING FOR HUMAN CD31 AND α-SMA ● TIMING 4–5 D

(1) Dissect the plugs following the dissection protocol (PROCEDURE Steps 35–40).
(2) Cut the plugs into pieces of approx. 2 mm × 2 mm using a scalpel.
▲ CRITICAL STEP Every step must be performed at 4 °C. Incubation steps must always be supported by gentle agitation.
(3) Fix the plugs in methanol containing 25% DMSO for 24 h at 4 °C in a 50 ml reaction tube.
(4) Carefully aspirate the methanol/DMSO solution with a glass pipette.
(5) Wash the plugs three times (at least 30 min each time) with sterile PBS at 4 °C with gentle agitation.
■ PAUSE POINT The protocol may be interrupted at this point: the pieces of the plug can be stored in PBS with 0.1% sodium azide at 4 °C for up to one week.
(6) Incubate pieces of the plug for 3 h in 3% BSA in PBST at 4 °C under gentle agitation.
(7) Incubate pieces of the plug in blocking buffer (3% BSA in PBST) containing the primary mouse anti-human CD31 (JC70A) antibody (dilution: 1:100) overnight at 4 °C under gentle agitation.
(8) Wash the plugs three times with PBST for 3 h at 4 °C under gentle agitation.
(9) Incubate the plugs in blocking buffer (3% BSA in PBST) containing the secondary goat anti-mouse antibody (Alexa Fluor 488) in a 1:200 dilution at 4 °C overnight at 4 °C under gentle agitation.
(10) Wash the plugs at least three times in PBST for 3 h at 4 °C under gentle agitation.
▲ CRITICAL STEP The quality of the staining may be controlled at this point by using a fluorescence microscope to check the background of the staining. If the background is too high, wash the plugs again overnight at 4 °C under gentle agitation.
(11) Incubate the plugs in PBS containing the mouse-anti-human α-SMA antibody (dilution 1:100) overnight at 4 °C under gentle agitation.
(12) Wash the plugs three times for 3 h with PBST at 4 °C under gentle agitation.
(13) Check under the fluorescence microscope if you can detect proper staining.
(14) Embed the plugs in mounting medium.
(15) Seal the slides with pertex so that they cannot dry out.
■ PAUSE POINT Stained pieces of plug can be stored at 4 °C for up to 1 week.
(16) The three dimensional structure of the capillary network may be analyzed by confocal microscopy.

Dehydration and paraffin embedding ● TIMING ~19.5 h

41| Dehydrate in an ascending alcohol series (75% ethanol (1:45 h, RT); 85% ethanol (2:00 h, RT); 96% ethanol (2:00 h, RT); isopropanol (2:00 h, RT); isopropanol (2:00 h, RT); isopropanol (2:00 h, RT); xylol (2:00 h, RT); xylol (2:00 h, RT); paraffin (2:00 h, 60 °C); and paraffin (1:45 h, 60 °C)).

42| Embed tissues into paraffin blocks for sectioning.
! CAUTION The processing, embedding and sectioning of paraffin blocks needs specialized equipment and expertise, and is usually carried out in a histology or pathology laboratory. Although hand processing can be carried out by following several available protocols.
■ PAUSE POINT The protocol may be interrupted at this point: Paraffin embedded plugs may be stored at RT until sectioning.

Sectioning ● TIMING ~4 h

43| If the microtome has a water basin, set the temperature to 42 °C.

44| Set the temperature of a heating plate to 42 °C.

45| Fix the paraffin block into the microtome, cut the surface until the plug is reached.

46| Cut 5–7 μm thick paraffin sections, mount them on glass slides.
? TROUBLESHOOTING

47| Transfer the glass slides to a heating plate and allow them to adhere until the sectioning is over.

48| Put all glass slides in a storage box and place it in a 37 °C incubator to dry the slides overnight.
▲ CRITICAL STEP Slides must not be stored for longer than 12 h at 37 °C.
▲ CRITICAL STEP Make sure that the sectioning is carried out by controlling the sections under a microscope.
Unless the plug is reached during cutting, check the first sections under the microscope to ensure that the paraffin sections are being cut within the matrigel plug.
■ PAUSE POINT The protocol may be interrupted at this point: Paraffin sections may be stored at RT for ~6 months.

Staining of sections for microscopic analysis

49| Sections can be stained and analyzed microscopically using option A for double-immunofluorescence staining for human CD34 and α-SMA, option B for double-immunofluorescence staining for human CD34 and mouse CD31 or option C for 3.3′-DAB staining for human CD31.

(A) Double-immunofluorescence staining for human CD34 and α-SMA ● **TIMING** ~6–7 h (for quantitative analysis)

(i) Deparaffinize and rehydrate the tissue sections by using a series of alcohol (xylol (5 min, RT); xylol (5 min, RT); ethanol (99%) (5 min, RT); ethanol (80%) (5 min, RT); ethanol (70%) (5 min, RT); and H_2O (at least 1 min, RT).

(ii) Samples may be encircled by a liquid barrier marker.

(iii) Pipette 60 µl goat sera on every section (blocking solution, ready-to-use).
▲ **CRITICAL STEP** The sections must never dry out during the entire procedure.

(iv) Incubate the slides in a wet chamber (plastic box with a wet tissue inside that can be closed) for 1 h at RT.

(v) In the meantime, dilute (1:50) the primary anti-human CD34 antibody in blocking solution.

(vi) One slide should serve as a negative control and should be incubated with blocking solution instead of the primary antibody.

(vii) Aspirate the blocking solution from the sections.

(viii) Pipette 60 µl of primary antibody solution on top of each section.

(ix) Incubate the slides for 2 h at RT in a wet chamber.

(x) Remove all solutions from the samples by tapping the slides on a paper and put the slides into a beaker with TBST.

(xi) Wash three times for 5 min with gentle agitation.

(xii) In the meantime, dilute the secondary anti-mouse IgG antibody (Alexa Fluor 488, 1:200) in PBS.

(xiii) Pipette 60 µl of the secondary antibody solution on each sample and incubate in a wet chamber for 45 min at RT.
▲ **CRITICAL STEP** Protect fluorescence labelled antibodies from light.

(xiv) Remove the secondary antibody solution from the sections by tapping the slides on a paper and put the slides into a beaker with TBST.

(xv) Wash the slides with TBST (three times for 5 min with gentle agitation).

(xvi) In the meantime, dilute the Cy3 labelled anti-α-SMA antibody (1:100) in PBS. Add Hoechst dye (dilution: 1:5,000).

(xvii) Pipette 60 µl of the antibody solution on each sample.

(xviii) Incubate the slides in a wet chamber for 1 h at RT.

(xix) Remove the antibody solution by tapping the slides on a paper and put the slides into a beaker with TBST.

(xx) Wash the slides with TBST (three times for 5 min with gentle agitation).

(xxi) Remove excess TBST from the samples by tapping the slides on a paper and cover them with mounting medium. Put a coverglass on each slide.
■ **PAUSE POINT** The protocol may be interrupted at this point: Slides may be stored at 4 °C in the dark, for ~4 weeks. Allow the mounting medium to polymerize (overnight at 4 °C) before the microscopic examination.
? TROUBLESHOOTING

(B) Double-immunofluorescence staining for human CD34 and mouse CD31 ● **TIMING** ~3 h

(i) Deparaffinize and rehydrate the tissue sections by using a series of alcohol (xylol (5 min, RT); xylol (5 min, RT); ethanol (99%) (5 min, RT); ethanol (80%) (5 min, RT); ethanol (70%) (5 min, RT); and H_2O (at least 1 min, RT)).
▲ **CRITICAL STEP** Replace the alcohol series after the use of ~1,000 slides. Do not extend the aforementioned incubation times (see Step 49BI).

(ii) Frame each of your sections with a liquid barrier marker.

(iii) Pipette 60 µl proteinase-K solution (8 µg ml^{-1} in TE buffer) on each section.
▲ **CRITICAL STEP** The sections should never dry out during the entire procedure.

(iv) Incubate the slides for 10 min at 37 °C in a wet chamber in an incubator.

(v) Remove the proteinase-K solution by tapping the slides on a paper and rinse the slides with TBST (two times for 5 min with gentle agitation).

(vi) After washing, pipette 60 µl goat sera (blocking solution, ready-to-use) on each section.

(vii) Incubate the slides for 30 min at RT in a wet chamber.

(viii) Meanwhile, prepare both primary antibodies in one reaction tube: mouse anti-human CD34 (QBEND10) in a 1:50 dilution and rat anti-mouse CD31 in a 1:100 dilution in the blocking solution.

(ix) Leave the blocking solution on the slide that should be the negative control.

(x) Aspirate the blocking solution from the rest of the sections.

(xi) Pipette 60 µl of the primary antibody solution on top of the slides.

(xii) Incubate the slides for 30 min at RT in a wet chamber.

(xiii) Remove all solutions from the sections by tapping the slides on a paper and put the slides in a beaker with TBST.

(xiv) Rinse the slides with TBST (three times for 5 min with gentle agitation).

(xv) In the meantime, prepare the secondary antibodies in one reaction tube: goat anti-rat Alexa Fluor 488 and goat anti-mouse Alexa Fluor 546 in a 1:500 dilution in PBS.
(xvi) Take the slides out of the wet chamber and pipette 60 µl secondary antibody solution on top of every section.
▲ CRITICAL STEP Fluorescent dye e.g., Alexa Fluor 488 is photosensitive therefore use a humidity chamber that is protected from light.
(xvii) Incubate for 30 min at RT in a humidity chamber.
(xviii) Remove the secondary antibody solution from the sections by tapping the slides on a paper and put the slides in a beaker with TBST.
(xix) Rinse the slides with TBST (three times for 5 min with gentle agitation).
(xx) Prepare Hoechst dye in a 1:5,000 dilution in PBS.
(xxi) Take the slides out of the wet chamber and pipette 60 µl of the Hoechst dye solution on top of every section of each slide.
(xxii) Incubate for 5 min at RT in a wet chamber.
(xxiii) Remove the Hoechst solution from the sections by tapping the slides on a paper and put the slides in a beaker with TBST.
(xxiv) Wash the slides with TBST (three times for 5 min with gentle agitation).
(xxv) Remove the slides, dry them slightly and cover the sections with fluorescent mounting medium and put a coverglass on each slide.
■ PAUSE POINT The protocol may be interrupted at this point. Slides may be stored at 4 °C in the dark for ~4 weeks. Allow the mounting medium to polymerize (overnight at 4 °C) before microscopic examination.
? TROUBLESHOOTING

(C) 3.3′-DAB staining for human CD31 ● TIMING ~12 h

(i) Deparaffinize and rehydrate the tissue sections by using a series of alcohol (xylol (5 min, RT); xylol (5 min, RT); ethanol (99%) (5 min, RT); ethanol (80%) (5 min, RT); ethanol (70%) (5 min, RT); and H_2O (at least 1 min, RT)).
▲ CRITICAL STEP Replace the alcohol series after the use of ~1,000 slides. Do not extend the aforementioned incubation times (see Step 49Ci).
(ii) Incubate the slides in 3% hydrogen peroxide (H_2O_2) in H_2O (vol/vol) for quenching of endogenous peroxidase activity.
▲ CRITICAL STEP H_2O_2 solution must be freshly prepared.
(iii) Wash the slides in H_2O two times for 3 min each in a beaker with gentle agitation.
(iv) Samples may be encircled by a liquid barrier marker.
(v) Place one drop of avidin solution on each section.
▲ CRITICAL STEP Sections must never dry out during the entire procedure.
(vi) Incubate the slides in a wet chamber for 10 min at RT.
(vii) Remove the avidin solution by tapping the slides on a paper and rinse the slides with PBS (1×) (two times for 5 min with gentle agitation).
(viii) Place one drop of biotin solution on each section.
(ix) Incubate for 10 min at RT in a wet chamber.
(x) Remove the biotin solution by tapping the slides on a paper and rinse the slides with PBS (1×) (two times for 5 min with gentle agitation).
(xi) In the meantime, prepare one drop of mouse on mouse (MOM) mouse IgG blocking reagent in 1.25 ml PBS.
(xii) After washing, pipette 60 µl of the MOM mouse IgG blocking reagent on each section.
(xiii) Incubate the slides for 1 h at RT in a wet chamber.
(xiv) Remove the blocking reagent from sections by tapping the slides on a paper and put the slides in a beaker with PBS.
(xv) Rinse the slides with PBS (three times for 5 min with gentle agitation).
(xvi) In the meantime, prepare 300 µl MOM diluent protein concentrate in 3.75 ml PBS.
(xvii) Pipette 60 µl of MOM diluent protein concentrate on each slide.
▲ CRITICAL STEP The remainder of the MOM diluent protein concentrate will be needed for the next day. Store it at 4 °C for further use.
(xviii) Incubate MOM diluent protein concentrate for 5 min at RT.
(xix) Prepare mouse anti-human CD31 (JC70A) in a 1:40 dilution in MOM diluent protein concentrate.
(xx) Leave the blocking solution on the slide that should be the negative control.
(xxi) Aspirate the blocking solution from the rest of the sections.
(xxii) Pipette 60 µl of the primary antibody solution on top of the slides.
(xxiii) Incubate the slides overnight at 4 °C in a wet chamber.
(xxiv) Remove the primary antibody by tapping the slides on a paper and rinse the slides with PBS three times for 5 min in a beaker with gentle agitation.

(xxv) In the meantime, prepare Vectastain Elite ABC Reagent in a ratio of one drop solution A and B in 1.25 ml PBS.
(xxvi) Incubate Vectastain Elite ABC Reagent for 30 min at RT.
(xxvii) In the meantime, dilute 5 µl MOM anti-mouse IgG biotin reagent in 1.25 ml MOM diluent protein concentrate.
(xxviii) After washing, pipette 60 µl MOM anti-mouse IgG biotin reagent on each section.
(xxix) Incubate the slides for 10 min at RT in a wet chamber.
(xxx) Remove the MOM anti-mouse IgG biotin reagent by tapping the slides on a paper, and rinse the slides with PBS for three times (5 min each) in a beaker with gentle agitation.
(xxxi) After washing, pipette 60 µl Vectastain Elite ABC Reagent on each section.
(xxxii) Incubate the slides for 5 min at RT in a wet chamber.
(xxxiii) Remove the Vectastain Elite ABC Reagent and rinse the slides with PBS for three times (5 min each) in a beaker with gentle agitation.
(xxxiv) In the meantime, prepare liquid 3.3′-DAB in a dilution of one drop DAB chromogen in 1 ml DAB substrate buffer.
! CAUTION DAB has been classified as a potential carcinogen and must be handled with care.
▲ CRITICAL STEP Transformation of DAB can rapidly occur depending on the staining intensity and should be monitored under the microscope.
(xxxv) Pipette 60 µl of the DAB solution on each section for several seconds. Observe under the microscope the substrate reaction and staining intensity.
(xxxvi) Stop the enzymatic DAB reaction at an adequate level by placing the slides in a beaker with H_2O.
(xxxvii) Wash the slides for 5 min at RT.
(xxxviii) Counterstain with freshly filtered haematoxylin at a dilution of 1:10 with distilled water.
! CAUTION Haematoxylin is toxic.
▲ CRITICAL STEP Haematoxylin must be filtered before use.
(xxxix) Incubate for 30 s at RT.
(xl) Transfer the slides in a chamber with tap water and incubate them under flowing tap water for 5 min at RT.
(xli) Dehydrate the samples in a series of alcohol (H_2O (1 min, RT); ethanol (70%) (5 min, RT); ethanol (80%) (5 min, RT); ethanol (99%) (5 min, RT); xylol (99%) (5 min, RT); and xylol (99%) (5 min, RT)).
▲ CRITICAL STEP Replace the alcohol series after ~1,000 slides have been rehydrated. Do not extend the aforementioned incubation times (see Step 49Cxli). Cover the slides with pertex and put a coverglass on each slide.
■ PAUSE POINT The protocol may be interrupted at this point: Store the slides at RT for ~6 months. Allow pertex to polymerize (overnight at RT) before examining the samples under a microscope.

Microscopy and statistical analysis

50| The plugs should be completely sectioned for thorough microscopic analysis; choose 3–4 slides from each plug (from the beginning, the middle and the end of each construct).

51| Determine the microvessel density (MVD) using slides stained for hCD34 (Step 49A and 49B). Images of the complete matrix area (magnification: 200×) should be taken, using a fluorescence microscope. We used an Olympus IX81 inverted microscope and individual images were assembled by multiple image alignment (Cell-P, Olympus, Germany).

52| Count all fluorescent vessels that have formed by hand and calculate them as vessel number per mm^2.

53| To analyze pericyte coverage, do a double-staining for hCD34 and α-SMA (Step 49A) and count the number of hCD34-positive vessels associated with α-SMA-positive mural cells (magnification: 400×).
? TROUBLESHOOTING

● TIMING

Steps 1–8, spheroid production: ~3 h
Steps 9–20, harvesting of spheroids: ~3 h
Steps 21–31, injection in the animal facility: ~1 h
Steps 32–40, dissection of implanted plugs: ~1.5 h
Box 1, whole mount staining for human CD31 and α-SMA: 4–5 d
Steps 41–42, dehydration and paraffin embedding: 19.5 h
Step 43–48, tissue slice sectioning: ~4 h
Step 49A(i–xxi), double-immunofluorescence staining for human CD34 and α-SMA: ~6–7 h
Step 49B (i–xxv), double-immunofluorescence staining for human CD34 and mouse CD31: ~3 h
Step 49C (i–xli), 3,3′-DAB staining for human CD31: overnight
Steps 52–55, microscopy and statistical analysis: depending on individual investigation

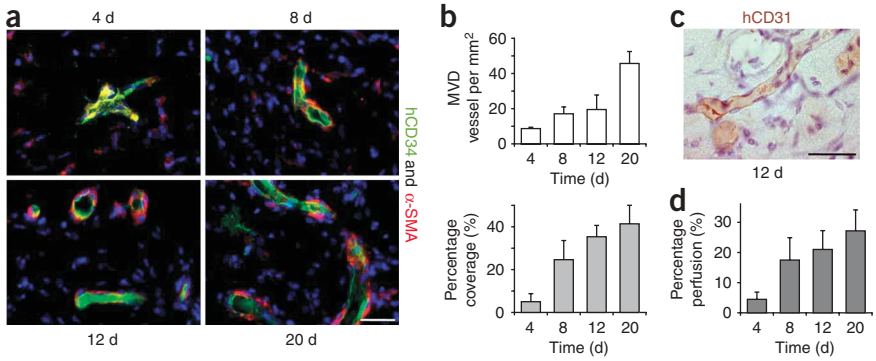

Figure 4 | Human neovessel formation originating from transplanted endothelial cell (EC) spheroids. (**a**) Validation of human neovessel formation after subcutaneous injection of human umbilical vein endothelial cell (HUVEC) spheroids in a matrigel–fibrin matrix containing vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) over time. Double immunofluorescence staining for grafted HUVEC with human CD34 (green) and mouse α-smooth muscle actin (α-SMA; red). Nuclei are counterstained with Hoechst 33258. Scale bar, 50 μm. (**b**) Quantification of microvessel density (MVD) and percentage of mural cell coverage (human CD34 (hCD34)-positive vessels that co-localize with α-SMA–positive host mural cells). (**c**) Immunoperoxidase staining of human CD31 (hCD31)-positive grafted HUVEC with hematoxylin counterstain. Scale bar, 50 μm. (**d**) Percentage of perfusion (hCD31-positive vessels containing RBCs) over time. The results are mean values ± s.d. for MVD and mural cell coverage and mean ± s.e.m. for perfusion analysis ($n = 5$)[15]. All animal procedures were carried out in accordance with the guidelines outlined by the local and national committees for animal experiments. Reprinted in part with permission from ref. 15.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Spheroid preparation (1–8)	Inadequate spheroid formation	Gain-/loss-of-function experiments may limit cell viability	Try to reduce the concentration of transfection reagent (e.g., for virus transfection the MOI (Multiplicity of infection = ratio of infectious virus particles to cells))
Harvesting of spheroids (18)	Spheroids cannot be sedimented	Insufficient centrifugation step	Centrifuge again 5 min at 500*g* at RT
Injection of spheroids (28)	Failed injection	Syringe needle is blocked	Spheroid/methocel/fibrinogen/ECBM mixture must not stand too long on ice
Plug dissection (38)	Plug could not be dissected	No bulge has been formed upon matrigel injection	Make sure to subcutaneously inject the matrigel/fibrin plug. Intraperitoneally administered matrigel cannot be recovered. During injection, the needle must always be visible through the skin. Move it gently to clear a space for the matrigel/fibrin fluid
Sectioning (46)	Ripped or damaged tissue sections	Blunt knife	Exchange the knife
		Temperature of the paraffin block too high	Ensure that the temperature of the paraffin block is kept low by cooling it on ice before sectioning
Staining procedure (CD34 and α-SMA; 49A) (mCD31 and hCD34; 49B)	Intense background fluorescence	Old fixative, over-extended fixation period	Fixative must be freshly prepared. The fixation of the tissue must not exceed 12 h at RT Usually, overnight incubation at 4 °C is adequate for complete fixation
Staining (49A, B)	Unspecific fluorescent particles on stained tissues	Alexa fluorochromes may precipitate	Spin down Alexa fluorochromes before use
Analysis (51–53)	Highly variable results	Inconsistent injection site	Choosing consistent injection sites is critical to achieve homogenous results. Responses to individual stimuli may significantly vary depending on the chosen site of injection
Analysis (51–53)	No vessel growth in some parts of the matrigel plug	Inhomogenous or inadequate subcutaneous injection.	After piercing the skin with the needle, move it gently to the left and right to clear some space for the matrigel mixture
			Inject the matrigel mixture by a firm continuous push of the syringe barrel

ECBM, endothelial cell basal medium.

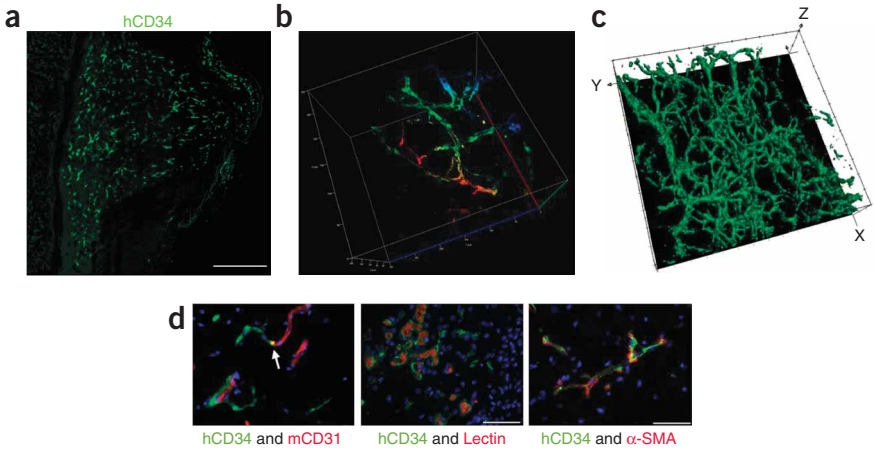

Figure 5 | Characterization of the human vascularization *in vivo* 20 d after implantation. (**a**) Overview of a whole slide, showing human CD34 (hCD34)-positive neovessels in a human umbilical vein endothelial cell (HUVEC) matrigel–fibrin plug (scale bar, 500 μm). (**b**,**c**) Three-dimensional structure of the human neovasculature as assessed by confocal microscopy using immunofluorescence whole-mount staining for hCD31 of a plug implanted for 20 d (slice 0.78 mm, *z* axis 36.5 mm). (**d**) Immunofluorescence images of human CD34-positive (hCD34) and mouse CD31-positive (mCD31) vessels (scale bar, 50 μm). The white arrow shows the side of an anastomose between a human and murine vessel (left). Mice were intravenously injected with lectin and the sections were immunofluorescence stained for hCD34 (middle). The human neovasculature was additionally analyzed using immunohistochemical staining for hCD34 and α-SMA (right). Nuclei in all images were counterstained with Hoechst 33258. Scale bar, 50 μm. All animal procedures were carried out in accordance with the guidelines outlined by the local and national committees for animal experiments. Reprinted in part with permission from ref. 15.

ANTICIPATED RESULTS

Plugs have been analyzed for the formation of a human EC-derived neovasculature 4, 8, 12 and 20 d after subcutaneous injection. Capillary tubes consisting of human EC form within 4 d of implantation when mural cell recruitment cannot yet be observed (**Fig. 4a**). After 8 d, the outgrowing human vasculature should form anastomoses with the mouse vasculature and around 20% of the vessels should be covered by host-derived mural cells (**Fig. 5c**) to finally become perfused, as evidenced by the presence of mouse erythrocytes, (**Fig. 4a**) or injected with FITC-dextran (**Fig. 5c**). Based on the procedure outlined in this protocol, the human vascular network is able to grow to around 50 vessels per mm^2 within 20 d (**Fig. 4b**), when ~40% are covered by mural cells, which can be confirmed by whole mount staining and confocal 3D image analysis (**Fig. 5b**)[15]. We have shown that the grown vasculature consists almost exclusively of human ECs and only few mouse ECs invade into the plug[15]. It is not known how long these implants can be stably maintained, but we have kept them for 60 d with no loss of phenotype (indeed, MVD increases over time). Although the assay has been optimized for HUVEC, other populations of human ECs may also be induced to form a functional neovasculature on xenotransplantion in immunocompromised mice by adapting this protocol, accordingly. In addition, we have shown that the assay is suitable for co-implantation experiments of ECs and TCs (**Fig. 6**)[15].

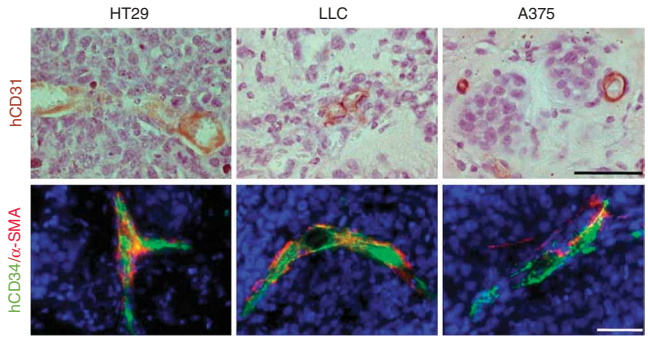

Figure 6 | Co-grafting of different tumor cell populations (HT29, colon cancer; LLC, Lewis lung carcinoma; A375, melanoma) with human umbilical vein endothelial cell (HUVEC) spheroids to show the contribution of co-grafted endothelial cell (EC) to tumor angiogenesis. Upper lane: Immunoperoxidase staining of human CD31 (hCD31)-positive grafted HUVEC with hematoxylin counterstain. Scale bar, 50 μm. Lower lane: Immunofluorescence images of human CD34-positive (hCD34), α-SMA-positive vessels. Scale bar, 50 μm. The grafted EC contribute with high efficacy to vascularization of grafted tumors. All animal procedures were carried out in accordance with the guidelines outlined by the local and national committees for animal experiments. Reprinted in part with permission from ref. 15.

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1. Adams, R.H. & Alitalo, K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat. Rev. Mol. Cell Biol.* **8**, 464–478 (2007).
2. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671–674 (1997).
3. Kerbel, R.S. Tumor angiogenesis. *N. Engl. J. Med.* **358**, 2039–2049 (2008).
4. Eichmann, A., Makinen, T. & Alitalo, K. Neural guidance molecules regulate vascular remodeling and vessel navigation. *Genes Dev.* **19**, 1013–1021 (2005).
5. Neufeld, G. & Kessler, O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis. *Nat. Rev. Cancer* **8**, 632–645 (2008).

6. Gerhardt, H. & Betsholtz, C. Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res.* **314**, 15–23 (2003).
7. Ellis, L.M. & Hicklin, D.J. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat. Rev. Cancer* **8**, 579–591 (2008).
8. Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* **438**, 932–936 (2005).
9. Augustin, H.G. *Methods in Endothelial Cell Biology* (Springer, Berlin, 2004).
10. Ausprunk, D.H., Knighton, D.R. & Folkman, J. Vascularization of normal and neoplastic tissues grafted to the chick chorioallantois. Role of host and preexisting graft blood vessels. *Am. J. Pathol.* **79**, 597–618 (1975).
11. Korff, T. & Augustin, H.G. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J. Cell Biol.* **143**, 1341–1352 (1998).
12. Norrby, K. *In vivo* models of angiogenesis. *J. Cell Mol. Med.* **10**, 588–612 (2006).
13. Passaniti, A. *et al.* A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab. Invest.* **67**, 519–528 (1992).
14. Jain, R.K., Schlenger, K., Hockel, M. & Yuan, F. Quantitative angiogenesis assays: progress and problems. *Nat. Med.* **3**, 1203–1208 (1997).
15. Alajati, A. *et al.* Spheroid-based engineering of a human vasculature in mice. *Nat. Methods* **5**, 439–445 (2008).
16. Wong, C., Inman, E., Spaethe, R. & Helgerson, S. Fibrin-based biomaterials to deliver human growth factors. *Thromb. Haemost.* **89**, 573–582 (2003).
17. Coughlin, S.R. Thrombin signalling and protease-activated receptors. *Nature* **407**, 258–264 (2000).
18. Kleinman, H.K. Preparation of gelled substrates. *Curr. Protoc. Cell Biol.* **Chapter 10**, Unit 10.3 (2001).