Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells

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Abstract (max 150 words)

Human endothelial cells (ECs) and pericytes are of great interest for research on vascular development and disease as well as future therapy. This protocol describes the efficient generation of ECs and pericytes from human pluripotent stem cells (hPSCs) under defined conditions. Essential steps for hPSC culture, differentiation, isolation and functional characterization of ECs and pericytes are described. Substantial numbers of both cell types can be derived in only 2-3 weeks: this involves differentiation (10 days), isolation (1 day) and 4 or 10 days expansion of ECs and pericytes, respectively. We also describe two assays for functional evaluation of hPSC-derived ECs: (i) primary vascular plexus formation upon co-culture with hPSC-derived pericytes and (ii) incorporation in the vasculature of zebrafish xenografts *in vivo*. These assays can be used to test the quality and drug sensitivity of hPSC-derived ECs and model vascular diseases with patient-derived hPSCs.

INTRODUCTION

The discovery of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSCs) was a major breakthrough for biomedical research[1-3]. The ability to generate many cell types of the human body made it possible to consider not only therapeutic applications for tissue regeneration but also disease modeling and drug discovery on human cells. Whilst initial challenges in deriving specific cell lineages through differentiation have been substantially addressed for neuronal, cardiac and endoderm cell types, including hepatic, lung and intestinal cells[4-11], the derivation of large numbers of healthy and patient specific endothelial cells (ECs) for use in pharmacological screening and tissue vascularization after transplantation has remained difficult. In addition, since dysfunctional ECs have been linked to multiple genetic and metabolic vascular disease conditions and the growth of tumors is promoted by neovascularization, new human cellular systems that model these conditions could prove crucial to moving these fields forward.

For decades, human umbilical vein endothelial cells (HUVEC) have been the "gold standard" in EC research because they are easily accessible from surplus umbilical

cords. More recently, endothelial progenitor cells (EPCs) derived as endothelial colony forming cells (ECFCs) from cultured cord- or adult peripheral blood have also been used as a source of ECs[12,13]. Although cord blood banking is valuable for collecting hematopoietic stem cells, cord blood is not available from all individuals and it is not amenable to scaled up production, thus alternative sources for patient specific ECs would be of value.

The importance of pericytes in the formation and function of the vasculature has been recognized for some time. Several studies demonstrated their role in regulating EC "sprouting", potentiating the formation of an EC barrier, determining blood capillary diameter, as well as in overall blood capillary stability[14]. Pericyte loss has been associated with multiple neurovascular diseases, including diabetic retinopathy, brain hemorrhage and some neurodegenerative disorders, as well as a target of some cardiotoxic drugs[15-17]. More recently, pericytes have also emerged as a possible source of adult multipotent mesenchymal stem cells (MSCs)[18], that can contribute to the regeneration of not only blood vessels, but also bone, cartilage, fat and skeletal muscle[19,20].

We have shown recently that it is possible to derive the principle vascular cell types robustly from human pluripotent stem cells (hPSCs), and that the cells can be expanded in culture and cryopreserved and are functional *in vitro* and *in vivo*[21]. Here, we describe these fully defined protocols for EC and pericyte derivation and functional analysis. hPSCs as used in these protocols are a renewable source of healthy and patient specific vascular cells, enabling many of the applications mentioned above to be developed further. Figure 1 gives an over view of the entire procedure.

Overview of previous methods to derive ECs and pericytes from hPSCs

Several independent laboratories have published descriptions of the derivation of ECs and pericytes from hPSCs [22,23]. However, these have often been difficult to reproduce due to neither the culture nor differentiation conditions for hPSC being standardized using fully defined reagents. Multiple factors can affect the outcome of differentiation: the presence of feeder cells, fetal bovine serum (FBS), bovine serum albumin (BSA) and the quality of stromal cell lines and growth factors. Most of the earliest protocols were developed based either on serum-supplemented media or stromal cell lines, or both[22-28]. Although defined conditions have recently been described, they result in very low efficiencies of EC differentiation (<1.5% ECs)[29], and are difficult to scale up due to their methodology being based on aggregate (or embryoid body (EB)) formation[30-32]. More recently, an efficient forced aggregation (or so-called SpinEB) method for EC differentiation has been developed[33] but this has the significant disadvantage that an additional step is required in which hPSCs are adapted to single cell passage. Establishing single cell passage can be time consuming and difficult to perform for multiple hPSC lines simultaneously. An alternative, technically more straightforward monolayer differentiation method has been used, however neither the percentages of ECs in the differentiated cell populations nor evidence that the protocol was robust across multiple hPSC lines was provided[34].

In addition to efforts to develop EC differentiation protocols, methods for the differentiation of pericytes and SMCs (or mural cells) from hPSCs, have been recently established[23,27,35], although these do not describe simultaneous differentiation of ECs and mural cells in defined conditions that facilitate efficient purification and expansion of both cell populations in one procedure. Further, expansion and cryopreservation without loss of functionality is a critical requirement for successful application of hPSC-derived ECs; our recent paper was the first in which this has been described [21].

Experimental rationale for the derivation of EC and pericytes.

Source hPSC and initial passage (steps 1-12). The protocol described here has been optimized for hPSCs maintained in commercially available and well-defined mTeSR™1 stem cell growth medium and passaged enzymatically. The differentiation protocol was tested and simplified for use on multiple hPSC-lines, including various human embryonic stem cell lines (NL-HES4, HES3 (NKX2-5eGFP/w)), as well human induced pluripotent stem cell lines derived from either skin fibroblasts or blood outgrowth endothelial cells (BOECs) by transduction with either retro- or lentiviruses. The differentiation efficiency was indistinguishable among more than 15 different hPSC lines tested in our laboratory (V.V.O., unpublished)[21]. The differentiation was induced in BPEL medium (Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids)[36], described previously where Polyvinylalchohol is omitted B(P)EL or the animal product-free equivalent (APEL), which is commercially available from Stemcell Technologies™.

Differentiation to EC (steps 13-15). The first mesoderm initiation step is based on previously published cardiac mesoderm induction from hPSCs, modified using information from our (unpublished) findings on vascular specification in SpinEBs[8]. More specifically, hPSCs are maintained on Matrigel™-coated plates in mTeSR™1 pluripotent stem cell culture medium and routinely enzymatically passaged once per week (**Fig. 2**). On average, one hPSC colony can be divided into 8-10 pieces (**Fig. 2a,b,c**) and 5-8 pieces distributed over one well of a 6-well plate prior to the induction of differentiation. One well of hPSCs is thus sufficient to make 2-3 6-well plates (12-18 wells) ready for differentiation. Differentiation is induced four days after passaging (day 0)(**Fig. 2d**). Mesoderm specification is induced by addition of bone morphogenetic protein 4 (BMP4), activin A, small molecule inhibitor of glycogen synthase kinase (GSK)3β (CHIR)[37] and vascular endothelial growth factor (VEGF). Upon mesoderm induction, pluripotent colonies continue to increase in size and mesenchymal cells are observed at day 3 of differentiation **(Fig. 2e, f)**. Importantly, the presence of activin A on days 0-3 resulted in more robust induction of ECs among different hPSC lines (unpublished). Mesoderm inductive factors are removed on day 3 of differentiation and replaced with vascular specification medium supplemented with VEGF and the transforming growth factor β (TGFβ) pathway small molecule inhibitor SB431542. SB431542 specifically inhibits activin receptor-like kinase -4,-5,-7 type I receptors (ALK-5, -4, -7) and supports expansion of ECs by inhibiting anti-proliferative activities of cell-derived TGFβ-like factors also present in the culture. The earliest ECs can be observed on day 6-7 of differentiation (**Fig. 2i,j**). Vascular specification medium is additionally refreshed on day 7 and day 9 of differentiation, and isolation of ECs is performed on day 10. Day 10 of

differentiation was chosen on the basis of morphological and immunophenotype examination of the differentiated cultures, to identify the timing that results on the highest percentages and total cell yield of mature vascular endothelial (VE)-Cadherin/platelet endothelial cell adhesion molecule (PECAM-1, also termed CD31) positive ECs (**Fig. 3**). Day 10 of differentiation is also more robust for the isolation of ECs should differentiation be delayed.

Isolation and expansion of EC (steps 16-29 and step 30 option A). ECs are isolated using a simple procedure of immunomagnetic selection with anti-CD31 antibody coupled magnetic beads (Dynabeads). Upon isolation, ECs are transferred to endothelial cell serum free medium (EC-SFM) to which platelet poor plasma serum (1%), VEGF and basic fibroblast growth factor (bFGF) have been added as described previously for derivation of blood brain barrier ECs from hPSCs[34]. Immediately after isolation, ECs are highly proliferative and tend to reach confluence within 2 or 3 days of plating if seeded at $5x10^3$-$10x10^3$ cells/cm^2.

Derivation of pericytes (step 30, option B). If desired, pericytes can be derived from the CD31- fraction simultaneously with the isolation of ECs. Pericytes thus derived are initially expanded in endothelial growth medium-2 (EGM-2), and then transferred to pericyte differentiation medium consisting of DMEM-10% FBS supplemented with TGFβ and platelet-derived growth factor (PDGF)-BB for 3 days. After 3 days, pericytes are switched to the maintenance medium consisting of DMEM-10% FBS.

Characterisation of EC and pericytes

At this stage of the procedure culture of the ECs can be continued by re-plating with a split ratio of 1:3 to 1:4. Immunotyping should be implemented or their phenotype should be confirmed (step 31, option A and B), or cells can be cryopreserved (step 31, option C). hPSC-derived ECs should display typical endothelial morphology with junctional localization of VE-cadherin, CD31 and von Willebrand factor (vWF) expression and the absence of smooth muscle actin (SMA) (**Fig. 4a,c**).

Pericytes are routinely maintained in DMEM-10% FBS and at this stage in the procedure can also be additionally passaged, cryopreserved or characterized. At this stage, pericytes appear as a homogenous population of cells (**Fig. 4b**). Upon culture in DMEM-10% FBS and stimulation with TGFβ and PDGF-BB for 3 days, pericytes upregulate expression of some smooth muscle markers, such as smooth muscle-specific protein 22 (SM22), Caldesmon and show heterogeneous expression of alpha smooth muscle actin (SMA) (**Fig. 4d**).

Assessment of EC functionality

Whilst morphological and phenotypic characterization of ECs and pericytes is required to confirm the identity and purity of the isolated population (step 31), this step does not address the functionality of the isolated cells. For many years, the Matrigel tube formation assay has been used as a standard for assessing EC functionality *in vitro*. However, this assay has a number of disadvantages and does not always confirm EC identity since many other cell types, including mesenchymal cells, are capable of forming tubes on top of Matrigel. Another drawback of this assay

and others is the inability to assess whether pericyte/mesenchymal cell interactions are functional *in vitro*. In addition, although mouse models have been developed to evaluate EC, pericyte and SMC functionality *in vivo*, these are time-, cost- and labor consuming and hard to adapt for drug screening and disease modeling applications. Additionally, these methods are incompatible with implementing the 3Rs in animal experimentation (reduce, refine and replace).

We describe two independent functional assays that can be used to address these issues and test vascular cell functionality. Firstly, the co-culture of hPSC-derived ECs with hPSC-derived pericytes, which can be used to test the functionality of both cell types *in vitro.* Secondly, a xenograft model in zebrafish that can be used to test the functionality of hPSC-derived ECs *in vivo.*

Co-culture of hPSC-derived ECs with pericytes (step 32, option A): The co-culture assay is performed with ECs and pericytes derived from hPSCs. The assay is based on a previously published assay in which HUVECs were co-cultured with pericytes[38-40]. We suggest using HUVECs as a positive control for endothelial cells in the co-culture experiment. In addition, human pulmonary artery vascular smooth muscle cells (PA-vSMC) can be used as a positive control for stromal cells/ pericytes as suggested in the original report on co-culture with HUVEC[39]. However, we noticed that hPSC-derived ECs perform better in the co-culture assay than HUVECs.

Upon co-culture, hPSC-derived ECs first adhere to the substrate and form EC islands surrounded by pericytes that remodel into vascular-like structures. This recapitulates essential steps of primary vascular plexus formation. The formation of a vascular network is highly dependent on VEGF and TGFβ derived from mural cells[21,40]. We have demonstrated that inhibition of TGFβ receptor signaling pathway with SB431542 results in dramatic enhancement of EC proliferation and sprouting. The EC sprouts induce a contractile phenotype in the co-cultured pericytes. We have further shown that this is Notch signaling dependent[21]. This assay can be useful in studying EC remodeling and endothelial-pericyte interactions.

ECs can be visualized in co-cultures by immunocytochemical staining with either anti-CD31 or VE-cadherin specific antibodies; proliferative cells are visualized with the anti-Ki67 antibody and contractile pericytes are visualized with anti-SM22 antibody. Moreover, the EC network and the number of branches can be easily quantified with freely available software, such as AngioTool[41] (**Fig. 5a,b**). In addition, the number of proliferative nuclei and area covered by SM22 positive pericytes can also be quantified with a custom developed pipeline based on the CellProfiler software[42] (**Fig. 5c,d**).

Zebrafish xenograft as a model to assess mammalian EC functionality (step 32, option B):
Zebrafish xenotransplantation has recently emerged as a valuable assay for assessing tumor cell behavior *in vivo* and is now being widely used in human tumor studies, for example to model cancer cell metastasis and *in vivo* cancer drug discovery[43-45].
Zebrafish have multiple advantages:

- They are transparent so that vasculature is easily visualized in transgenic strains in which all blood vessels are genetically marked to express green fluorescent protein (GFP)(Tg(fli1:GFP))[46].
- Transplantation of human cells into early embryos does not require immunosuppression since the immune system only forms in late development. As a result, xenotransplantation has already been successful using different (mammalian and non-transgenic zebrafish cell types)[47-50].
- Imaging can be performed either on live embryos or fixed embryos.
- Injection of cells and imaging can also potentially be automated[51].

One of the most crucial functional features of ECs is their ability to form lumenized blood- and lymphatic vessels through which blood or fluid can flow. In addition, in the case of transplantation, it is important that any blood vessels that form can connect or anastomose with the host vasculature. The ability to visualize formation of vasculature (preferably in a living organism) is then an important aspect of assessing full functionality of any prospective ECs. We have demonstrated that zebrafish xenograft meets these requirements and that it can be used successfully to study EC functionality *in vivo*[21]. In addition, we found that hPSC-derived ECs perform better in zebrafish xenografts than HUVEC, so in principle HUVEC can be used as a negative control. We have not observed incorporation of pericytes into the zebrafish host vasculature.

The assay can be set up relatively easily in a lab that has access to a zebrafish facility which can provide zebrafish embryos; most of the other steps can be adapted to be carried out in a standard cell culture laboratory with two incubators (set at 28°C and 33°C respectively), a good quality stereo microscope, picopump and manual manipulator (**Fig. 6**). In addition, the assay requires relatively small amounts ($\sim 2.5 \times 10^5$) of fluorescently labeled cells, compared to the $\sim 10 \times 10^6$ cells needed for routine transplantation in a mouse.

Furthermore, excellent on-line resources are available for researchers with detailed descriptions of the zebrafish and embryo manipulation steps (https://wiki.zfin.org/display/prot/ZFIN+Protocol+Wiki).

The zebrafish xenograft assay can be performed in 10 days and includes the following steps:

- Steps i-iv: 24 hours to set up breeding and to collect fertilized eggs
- Steps v-vii: sorting of viable embryos at ~6 and 24 hours post fertilization (hpf); this can be performed manually or adapted to automated sort ;
- Step viii: sorting of embryos that express GFP 24 hpf (this can be performed manually or adapted to automated sort);
- Step x: dechorionation (chorion removal) of embryos (**Fig. 6a,b,c**);
- Steps xi-xxiii: fluorescently labeled ECs are injected into the duct of Cuvier (**Fig. 6d,e**);
- Steps xxvii-xxviii: visualization of EC incorporation into the host vasculature 5 days post injection (**Fig. 7**). This step can be also adapted to automated imaging.

Zebrafish embryos can be imaged live, or as fixed preparations for prolonged storage or additional immunocytochemical analysis of cells.

MATERIALS

REAGENTS

- hPSC: NL-HES4[52], HES3 (NKX2-5eGFP/w)[8], BOECs-iPSCs, Fib-iPSCs[53,54]
- BD Matrigel Matrix GFR (BD Biosciences, cat. no. 354230)
- Dispase II, powder (GIBCO, cat. no. 17105-041)
- DMEM:F12 1:1 (GIBCO, cat. no. 31331-028)
- mTeSR™1 Complete Kit for hESC Maintenance (Stemcell Technologies, cat. no. 05852)
- Penicillin-Streptomycin; 5,000 U ml^{-1} (GIBCO, cat no. 15070-063)
- StemDiff APEL Medium (Stemcell Technologies, cat. no. 05210)
- IMDM, no phenol red (GIBCO, cat. no. 21056-023)
- Ham's F-12 Nutrient Mix, GlutaMAX™ Supplement (GIBCO, cat. no. 31765-027)
- PFHM-II 1X (GIBCO, cat. no. 12040-077)
- AG 501-X8(D) Resin (Bio-rad, cat. no. 142-6425)
- BSA (Sigma-Aldrich, cat. no. A3311)
- BovoStar' BSA (Bovogen Biologicals Pty Ltd, cat. no. BSAS 0.5)
- Chemically Defined Lipid concentrate (GIBCO, cat. no. 11905031)
- ITS-X (Insulin-Transferrin-Selenium-Ethanolamine, 100X)(GIBCO, cat. no. 51500-056)
- Mono-Thio Glycerol (a-MTG) (Sigma-Aldrich, cat. no. M6145-25ml)
- AA2P (L-Ascorbic acid 2-phosphate)(Sigma-Aldrich, cat. no. A8960)
- Glutamax-1 supplement (GIBCO, cat. no. 35050-038)
- BMP4, (Miltenyi Biotec, cat. no. 130-095-549)
- Activin A (Miltenyi Biotec, cat. no. 130-095-547)
- CHIR99021 (Tocris Bioscience, cat. no. 4423)
- VEGF165 (R&D Systems, cat. no. 293-VE)
- SB431542 (Tocris Bioscience, cat. no. 1614)
- Dynabeads® CD31 Endothelial Cell (Invitrogen, cat. no. 11155D)
- DMEM, high glucose, no glutamine (GIBCO, cat. no. 11960-044)
- PBS, Dulbecco's (GIBCO, cat. no. 14190-094)
- 1x TrypLE Select (GIBCO, cat. no. 12563029)
- EDTA disodium salt (Sigma-Aldrich, cat. no. E5134)
- FBS (GIBCO, cat no. 10270-106)
- EC-SFM (Human Endothelial-SFM)(GIBCO, cat. no. 11111-044)
- Platelet-poor plasma derived serum, bovine (Biomedical technologies inc, cat. no. BT-214)
- Human bFGF, premium grade (Miltenyi Biotec, cat. no. 130-093-842)
- Gelatin from porcine skin, type A (Sigma-Aldrich, cat. no. G1890)
- Distilled water (GIBCO, cat. no. 15230-089)
- EGM-2 Bulletkit (Lonza, cat. no. CC-3162).
- DMEM, high glucose (GIBCO, cat. no. 41966-052)
- TGFβ3 (Kenneth K. Iwata, OSI Pharmaceuticals, NY, USA)
- PDGF-BB (Peprotech, cat. no. 100-14B)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- 2-propanol (J.T.Baker, cat. no. 8067)
- PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich, cat. no. PKH67GL)
- Diluent C (Sigma-Aldrich, cat. no. CGLDIL-6X10ML**)**
- EBM-2 (Lonza, cat. no. CC-3156)
- Fibronectin (Sigma-Aldrich, cat. no. F1141)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787
- Paraformaldehyde (Merck, cat. no. 1.04005.1000) CAUTION Work in a fume hood!
- Sodium dihydrogen phosphate monohydrate; $NaH_2PO_4.1H_2O$ (Merck, cat. no. 1.06346.1000)
- Disodium hydrogen phosphate dehydrate; $Na_2HPO_4.2H_2O$ (J.T.Baker, cat. no. 0326)
- Normal Goat Serum (DAKO, cat. no. X0907)
- DAPI Nucleic Acid Stain, dilactate (Molecular Probes, cat. no. D3571)
- Instant Ocean® Sea Salts (Instant Ocean, cat. no. SS15-10)
- Methylene Blue (Sigma-Aldrich, cat. no. M9140)
- Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt) (Sigma, cat. no. 886-86-2)
- Cell Tracker Cm DiI (Invitrogen, cat. no. C7001)
- anti-human CD31-APC 1:100 (eBioscience, cat. no. 17-0319)
- anti-human PDGFRβ-PE 1:20 (BD Pharmigen, cat. no. 558821)
- anti-human VE-cadherin-A488 1:50 (eBioscience, cat. no. 53-1449)
- anti-human KDR-PE 1:20 (R&D systems, cat. no. FAB357P)
- anti-human CD31 1:200 (DAKO, cat. no. M082301)
- anti-human VE-cadherin 1:200 (CellSignal, cat. no. 2158)
- anti-human vWF 1:200 (DAKO, cat. no. A008202)

- anti-human SMA 1:200 (Sigma-Aldrich, cat. no. A2547)
- anti-human SM22 1:300 (Abcam, cat. no. ab14106)
- anti-human Caldesmon 1:200 (Sigma-Aldrich, cat. no. C4562)
- anti-human Ki67 1:50 (Abcam, cat. no. Ab833)
- Fluorescence mounting medium (DAKO, cat. no. S3023)

Step 32 option B only:
- Zebrafish Tg(fli1:GFP))[46]. CAUTION Zebrafish and embryos must be produced, staged, and maintained according to standard procedures compliant with the national and institutional animal welfare regulations.

EQUIPMENT

- Culture plates, 6 wells (Greiner Bio-One, cat. no. 657160)
- Hypodermic needle 26G Brown 13mm (BD Microlance, cat. no. 303800)
- Hypodermic syringe 1ml (BD plastipack, cat. no. 300013)
- Cell Scraper, blue28 cm (Greiner Bio-One, cat. no. 541070)
- CO2 cell culture incubator (Panasonic, cat. no. MCO-19AIC)
- CO2 cell culture incubator (SANYO Electric Co.,Ltd., cat. no. MCO-17A)
- Sterile biosafety cabinet (Cleanair)
- Leica MS5 stereomicroscope (Leica Mycrosystems)
- Stericup-GV 250 ml bottle with cap (Millipore, cat. no. SCGVU02RE)
- Puradisc™ 30 sterile syringe filter 0.2µm (Whatman, cat. no. 10462200)
- Hypodermic syringe 30ml (BD plastipack, cat. no. 301229)
- Hypodermic syringe 10ml (BD plastipack, cat. no. 302188)
- Tubes 50ml (Greiner Bio-One, cat. no. 227261)
- Tubes 10ml (Greiner Bio-One, cat. no. 188271)
- Sterile plastic pipette (Greiner Bio-One; 2 ml, cat. no. 710180; 5 ml, cat. no. 606180; 10 ml, cat. no. 607180; 25 ml, cat. no. 760180)
- Filter tips (Corning;10 µl, cat. no. 4807; 200 µl , cat. no. 4810; 1000 µl, cat. no. 4809)
- Pipetman starter kit P2, P10, P100 (Gilson international, cat. no. F167500S)
- Pipetman starter kit P20, P200, P1000 (Gilson international, cat. no. F167300)
- Dispenser Multipette® plus (Eppendorf, cat. no. 4981 000.019)
- T75 cell culture flask (Greiner Bio-one, cat. no. 658170)
- Sealing film 10cmx38m (Parafilm M, cat. no. 291-1213)
- Sterile filters 100µm (CellTrics, cat. no. 04-004-2328)
- DynaMag™-5 magnet (Life Technologies, cat. no. 12303D)
- DynaMag™-15 magnet (Life Technologies, cat. no. 12301D)
- BD Clay Adams™ Nutator mixer (BD Diagnostics, cat. no. 421105)
- Round-bottom tube 5 ml; FACS tube (BD Biosciences, cat. no. 352058)
- Cryo tubes (Greiner Bio-one, cat. no. 123263)
- Nalgene Cryo 1 °C freezing container (Thermo Scientific, cat. no. 5100-0001)
- Himac CT6EL centrifuge (Hitachi Koki Co., Ltd., cat. no. 905601)
- -80 °C Freezer MDF-U73V (SANYO Electric Co., Ltd., cat. no.
- Centrifuge (Eppendorf, cat. no. 5810R)
- Glass coverslips (Menzel-Glasser, cat. no. CB00140RA1)
- Imaging plate, 96-well (Corning, cat. no. 353219)
- BD Pathway™ 855 Bioimager (BD Biosciences, cat. no. 641760)
- Leica TCS SP5 Confocal Microscope (Leica Microsystems)
- Leica M50 stereomicroscope with a transmitted light-base (TL3000ST) (Leica Microsystems)
- Leica fluorescence stereomicroscope (MZFLIII) (Leica Microsystems)
- 28°C incubator (JIM Engineering, cat. no. GP-INC-100)
- 33°C incubator (JIM Engineering, cat. no. GP-INC-100)
- Petri dishes (Thermo Scientific, cat. no. 240401)
- Eppendorf microloader tips (Eppendorf, cat. no, 930001007)
- Plastic transfer pipettes (Sigma, cat. no. Z331767)
- 2X Jewellers forceps, Dumont No. 5 (Sigma, cat. no F6521-1EA)
- Stainless steel forceps (Sigma, cat. no. Z225304-1EA)
- SYS-PV820 Picopump with pressure, a foot switch (World Precision Instruments)
- Borosilicate glass capillary needles (1mm O.D. x 0.78mm I.D.)(Harvard Apparatus, cat. no. 300040)
- Zeiss Stemi DV4 stereomicroscope (Zeiss, cat. no. 435421-9901-000)

REAGENT SETUP

Dispase solution (5 mg ml^{-1}) Dissolve dispase at 5 mg ml^{-1} in DMEM/F12 by vortexing. Dissolve completely by incubation at 37 °C for 5 min and sterilize the solution using a 0.22 µm membrane filter. Prepare 1 or 2 ml aliquots and store for up to 1 year at -20°C.

Dispase solution (1 mg ml^{-1}) Thaw 5 mg ml^{-1} dispase solution. Dilute this solution 1:5 with DMEM/F12 to get a 1 mg ml^{-1} working concentration. Store for up to 2 weeks at 4 °C.

Gelatin solution (1% (wt/vol)) Add 1 g of gelatin to 100 ml of distilled water to make a 1% gelatin solution. Autoclave the solution and store 10 ml aliquots at -20 °C. For a 0.1% gelatin working solution, the stock solution is diluted with PBS and sterilized by filtration using a 0.22 µm membrane filter.

EDTA 0.5 M (pH 8.0) Add 186.1 g EDTA to 800 ml of distilled water. Adjust pH to 8.0 with NaOH and add distilled water to a final volume of 1 liter. Filter the solution through a 0.22 µm membrane filter and sterilize by autoclaving. Store for up to 1 year at room temperature (RT; 20°C).

FACS buffer Dissolve 1.25 g BSA in 250 ml PBS and add 1 ml of EDTA 0.5 M (pH 8.0). Sterilize the medium using a Stericup filter (0.22 µm) and store for up to 4 weeks at 4 ˚C.

FACSB-10 (FACS buffer-10% FBS) Add 5 ml FBS to 45 ml FACS buffer and sterilize by filtration using a 0.22 µm membrane filter. Store for 4 weeks at 4 ˚C.
DMEM-0.1% BSA Prepare 250 ml of DMEM/0.1% BSA. Dissolve 0.25 g BSA in 250 ml DMEM (high glucose, no glutamine). Sterilize the medium using a Stericup filter and store for 4 weeks at 4 ˚C.
PFA (8%) Add 40 g Paraformaldehyde to 400 ml Milli-Q water. Heat up to 60 ˚C and stir at medium speed. After a few min, add about 10 drops of 1 N NaOH to dissolve the paraformaldehyde granules. Eventually, the solution will become translucent. Let the solution cool down and add Milli-Q to a total volume of 500 ml. Store at 4 ˚C for up to 2 months. CAUTION Work in a fume hood!
Phosphate buffer 0.2 M (pH 7.4) Prepare solution 1; Dissolve 8.28 g $NaH_2PO_4.1H_2O$ in 300 ml Milli-Q. Prepare solution 2; Dissolve 10.78 g $Na_2HPO_4.2H_2O$ in 300 ml Milli-Q. Add solution 1 to solution 2 until pH 7.4 to make Phosphate buffer 0.2 M (pH 7.4). Can be stored at RT (no expiration date).
PFA (4%) Add 1 volume of PFA (8%) to 1 volume of 0.2 M Phosphate buffer (pH 7.4). Cover with foil and keep at 4 ˚C for up to 2 weeks. CAUTION Work in a fume hood!
PFA (1%) Dilute PFA (4%) 1:4 with PBS. Ideally prepare fresh or store at 4 ˚C for up to 1 week. CAUTION Work in a fume hood!
0.5%TX-100 in PBS Add 250 µl Triton® X-100 to 50 ml PBS. Sterilize by filtration using a 0.22 µm membrane filter. Store for up to 1 year at RT.
1%BSA (wt/vol) in PBS Dissolve 0.5 g BSA in 50 ml PBS. Sterilize by filtration using a 0.22 µm membrane filter and store for up to 4 weeks at 4 ˚C.
1%BSA (wt/vol)/2% goat serum (vol/vol) in PBS Add 10 µl goat serum to 500 µl PBS/1% BSA. Always prepare fresh.
1%BSA (wt/vol)/0.1% TX-100 (vol/vol) in PBS Dissolve 0.5 g BSA in 50 ml PBS and add 50 µl Triton® X-100. Sterilize by filtration using a 0.22 µm membrane filter and store for up to 4 weeks at 4 ˚C.
1%BSA (wt/vol)/0.1% TX-100 (vol/vol)/2% goat serum (vol/vol) in PBS Add 10 µl goat serum to 500 µl PBS/1% BSA/0.1% TX-100. Always prepare fresh.
10% BSA (wt/vol) Dissolve 10 g BSA (Bovostar) in 100 ml IMDM at 37 °C to prepare a 10% (wt/vol) stock solution. Sterilize by filtration using a 0.22 µm membrane filter and store for up to 4 weeks at 4 ˚C.
Deionized 10% BSA (wt/vol) Dissolve 10 g BSA (Sigma) in 100 ml IMDM at 37 °C to prepare a 10% (wt/vol) stock solution. Deionize by adding around 3 g of mixed resin beads to 100 ml of 10% BSA solution. Perform at least three steps of deionization at 4 ˚C (two hours each). Filter sterile and store for up to 3 month at 4 ˚C.
BMP-4 (30 µg ml^{-1} stock solution) Reconstitute the content of the vial to a concentration of 30 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store for up to 1 year at -80°C.
bFGF (100 µg ml^{-1} stock solution) Reconstitute the content of the vial to a concentration of 100 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store for up to 1 year at −80 °C.
Activin A (25 µg ml^{-1} stock solution)
Reconstitute the content of the vial to a concentration of 25 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store for up to 1 year at -80 °C.
CHIR (4mM) Reconstitute 10 mg in 5.37 ml in DMSO. Prepare aliquots and store at −20 °C.
VEGF (50 µg ml^{-1} stock solution) Reconstitute at 50 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store for up to 1 year at −80 °C.
SB431542 (20mM) Reconstitute 10 mg in 1.3 ml in DMSO. Prepare aliquots and store at −20 °C. No expiration date.
TGFβ3 (5 µg ml^{-1}) Reconstitute the content of the vial to a concentration of 5 µg ml^{-1} in 4mM HCl containing 0.1% (wt/vol) BSA. Prepare aliquots and store up to 1 year at −80 °C.
PDGF-BB (20 µg ml^{-1}) Reconstitute the content of the vial to a concentration of 20 µg ml^{-1} in PBS containing 1% (wt/vol) BSA. Prepare aliquots and store up to 1 year at −80 °C.
DAPI Dissolve 10 mg DAPI in 2 ml dH_2O to prepare a 5 mg ml^{-1} DAPI stock solution. Prepare aliquots and store for up to 6 months at 4 ˚C protected from light or at -20 ˚C for long term storage.
10x Tricaine Dissolve 400 mg Tricaine powder in 97.9 ml of deionized water, add ~2.1 ml 1 M Tris (pH 9). Adjust pH to ~7. Store aliquots at −20 °C. No expiration date.
Stock salt solution Dissolve 40 g Instant Ocean Sea Salts ™ in 1 L distilled water. Store at RT.
Egg water Add 1.5 ml of Stock salt solution to 1 L distilled water with a small amount of methylene blue to change the water color pale blue. Store at RT. No expiration date.

mTeSR™1 Prepare mTeSR™1 medium as shown. Make sure that the Lot. Nr. of **mTeSR™1** Basal medium and Supplement medium end with the same letter when ordering. Store for up to 2 weeks at 4°C or aliquot 40 ml in 50 ml tubes and store at -20 °C.

Composition	Volume	Final concentration
mTeSR™1 Basal Medium	400 ml (1 bottle)	
mTeSR™1 Supplement	100 ml (1 bottle)	
Pen/Strep (5,000 U ml^{-1})	2,5 ml	0.5%

B(P)EL Prepare 250 ml of B(P)EL as shown, no PVA. Sterilize the medium using a Stericup filter and store for up to 3 weeks. The formulation is based on previously described BPEL medium, where P is eliminated[21,36].

Composition	Volume	Final concentration
IMDM	107.63 ml	
F12	113.88 ml	
PFNMII	12.5 ml	5%
10% BSA in IMDM	6.25 ml	0.25%
Lipids (100X)	2.5 ml	1X
ITS-X (100X)	250 µl	1X
αMTG (13 µl in 1 ml IMDM)	750 µl	450 µM
AA2P (5 mg ml^{-1})	2.5 ml	0.05 mg ml^{-1}
Glutamax (200 mM)	2.5 ml	2 mM
Pen/Strep (5,000 U ml^{-1})	1.25 ml	0.5%

Mesoderm induction medium Prepare mesoderm induction medium as shown. Sterilize the medium using a 0.22 µm membrane filter. Always prepare fresh.

Composition	Volume	Final concentration
B(P)EL	20 ml	
Activin A (25 µg ml^{-1})	20 µl	25 ng ml^{-1}
BMP4 (30 µg ml^{-1})	20 µl	30 ng ml^{-1}
VEGF (50 µg ml^{-1})	20 µl	50 ng ml^{-1}
CHIR (4 mM)	7.5 µl	1.5 µM

Vascular specification medium Prepare vascular specification medium as shown. Sterilize the medium using a 0.22 µm membrane filter. Always prepare fresh.

Composition	Volume	Final concentration
B(P)EL	20 ml	
VEGF (50 µg ml^{-1})	20 µl	50 ng ml^{-1}
SB431542 (20 mM)	10 µl	10 µM

EC-SFM (FULL) medium Prepare 250 ml of EC-SFM medium as shown. Sterilize the medium using a Stericup filter and store for up to 2 weeks.

Composition	Volume	Final concentration
EC-SFM	250 ml	
Platelet-poor plasma derived serum	2.5 ml	1%
VEGF (50 µg ml^{-1})	150 µl	30 ng ml^{-1}
bFGF (100 µg ml^{-1})	50 µl	20 ng ml^{-1}

DMEM-10%FBS Prepare 250 ml of DMEM/10%FBS as shown. Sterilize the medium using a Stericup filter and store for up to 3 weeks.

Composition	Volume	Final concentration
DMEM, high glucose	225 ml	
FBS	25 ml	10%
Pen/strep (5,000 U ml^{-1})	1.25 ml	0.5%

DMEM-10% FBS + Growth factors Prepare DMEM-10% FBS + GF as shown. Sterilize the medium using a 0.22 µm membrane filter. Always prepare fresh.

Composition	Volume	Final concentration
DMEM-10%FBS	20	
TGFβ3 (5 µg ml^{-1})	8 µl	2 ng ml^{-1}
PDGF-BB (20 µg ml^{-1})	4 µl	4 ng ml^{-1}

EQUIPMENT SETUP

Matrigel-coated 6-well plate Thaw 120 µl Matrigel on ice. Transfer 12 ml cold DMEM/F12 to a 50 ml tube. Cool a pipet tip by pipetting cold DMEM/F12 up and down and transfer the Matrigel to the DMEM/F12. Mix and add 2 ml of Matrigel per 6-well or 20 µg cm^2 with a cooled pipet. Leave the plates at RT for 1h. Seal the plate with parafilm and store at 4 °C for up to 2 weeks. Warm the plates for 30 min at RT before use.

Gelatin-coated plate or flask Add 0.1% gelatin solution to a culture plate or flask. Make sure that the gelatin covers the whole surface. Use 1 ml of gelatin solution per 12-well plate, 2ml per 6-well, 4 ml per T25 flask and 10 ml per T75 flask. Leave the plate or flask with gelatin for at least 1 hour at 37 °C before use.

Fibronectin-coated glass coverslips Add 10 µl of fibronectin (1 mg ml^{-1}) to 2 ml PBS, end concentration is 5 µg ml^{-1}. Use 50 µl (~250 ng cm^2) of fibronectin solution per one cover slip in 12-well plate. Leave the plate with fibronectin for at least 1 hour at 37 °C before use.

Agarose plate Place 3 grams of agarose into a conical flask with 100 ml of egg water. Microwave at high heat stopping and swirling the mixture throughout the heating process until it is well dissolved (approximately 5 minutes). Cool until the solution is safe to handle and pour it into a Petri dish until the gel is approximately 5 mm thick. Cool at RT until set, and then transfer to a 4°C refrigerator. Agarose plates can be reused if kept air tight and stored at 4°C.

PROCEDURE

Passaging of hPSCs •TIMING 3h

1| Pre-warm Matrigel-coated plates, cell culture medium mTeSR™1, dispase solution and DMEM/F12 to RT.

▲ **CRITICAL STEP** All solutions have to be freshly prepared, see reagent setup for information regarding storage time.

2| Remove differentiated parts of the hPSC colonies by scraping them with a 10µl pipet tip.

3| Aspirate and discard mTeSR™1 and scraped differentiated parts.

4| Add 1 ml of dispase solution in DMEM/F12 (1 mg ml^{-1}), and incubate at 37°C for 3 min.

▲ **CRITICAL STEP** the timing of the enzymatic treatment varies, so it has to be monitored carefully. The dispase solution should not be added for a prolonged period of time, as it might affect the viability of hPSCs and the outcome of the differentiation experiment.

? **TROUBLESHOOTING**

5| Aspirate and discard the dispase solution.

6| Wash the wells three times with 2 ml of pre-warmed DMEM/F12. Do not aspirate DMEM/F12 from the last wash. Concurrently perform step 7.

▲ **CRITICAL STEP** The hPSC colonies should not be left in DMEM/F12 for a prolonged period of time, as continued incubation results in re-adhesion of colonies.

7| Prepare culture plates by aspirating the remaining Matrigel solution from the plates prepared in step 1 and adding 2 ml of mTeSR™1.

▲ **CRITICAL STEP** Matrigel should be aspirated prior to the addition of medium in order to avoid drying of the gel layer.

▲ CRITICAL STEP This step should be performed at the same time as step 6.

8| Scrape hPSC colonies off the plate with a cell scraper.

▲ **CRITICAL STEP** This step should be performed gently, without breaking colonies into pieces.

9| Collect pieces with a 1 ml tip and transfer them to a new 15-ml conical tube.

10| Gently break the pieces with a 1 ml pipet tip into small clumps. The size of the pieces should be ~500-1000 µm on average (Fig. 2b).

▲ **CRITICAL STEP** This step is crucial for hPSC viability, as small cell clumps will result in increased death, and bigger cell clumps will have a negative impact on the differentiation efficiency.

▲ **CRITICAL STEP** Various hPSC-lines behave differently with some dissociating more easily than others.

? **TROUBLESHOOTING**

11| Distribute 5-8 pieces per well into the 6-well Matrigel-coated plate from step 7 and place the plate in a 37°C incubator.

▲ **CRITICAL STEP** During this step it is important not to put more than 8 pieces per well. It is also crucial to avoid moving the plate whilst the pieces attach so that the pieces remain separated from each other.

Expansion of hPSCs prior to differentiation •TIMING 3-4 days

12| 24 h after passaging of hPSC-colonies, replace medium with 4ml of fresh mTeSR™1 and incubate colonies for a further 3-4 days **(Fig. 2c,d)**.

▲ **CRITICAL STEP** Routinely we do not observe much difference between incubating for a further 3 or 4 days prior to commencing differentiation. However, we would not advise expanding colonies for longer than 3-4 days. Timing is primarily dictated by the convenience of the differentiation starting point. As we routinely passage hPSC on Thursday, differentiation starts on Monday.

Monolayer differentiation of hPSCs •TIMING 10 days

13| ***Day (0) Mesoderm induction from hPSCs:*** Replace mTeSR™1 medium with 2ml of mesoderm induction medium and incubate cells for 2 days.

▲ **CRITICAL STEP Add freshly made up** growth factors to the differentiation medium.

14| ***Day (3) Vascular specification from hPSCs:*** replace mesoderm induction medium with 2ml of vascular specification medium and incubate for 4 days.

15| ***Day (7) and Day (9 or 10) Expansion of vascular cells:*** Replace medium with fresh vascular specification medium at day (7) and day (9) or day (10).

▲ **CRITICAL STEP** EC islands can be observed on Day (6-7) of differentiation (**Fig. 2i,j**). It is advisable to monitor differentiation efficiency both phenotypically and by flow cytometry, using the same procedure as that described in step 31| option A.

▲ **CRITICAL STEP** ECs can be isolated at Day 10-11 of differentiation. However, we do advise refreshing vascular specification medium one day prior to isolation of ECs.

? **TROUBLESHOOTING**

Dynabead isolation of ECs and pericytes •TIMING 3h

16| **Day 10 or 11:** Prepare gelatin-coated 6-well plates; FACS buffer and FACS buffer-10% FBS (FACSB-10), DMEM-0.1% BSA.

17| Calculate the total volume of CD31-Dynabeads needed, based on the following estimation: 7 µl beads per well of a 6-well plate.

▲ **CRITICAL STEP** We observed that ECs tend to tolerate 5-10 beads per cell well; thus a higher number of beads per cell is not recommended. The number of CD31-Dynabeads per ml is listed in the datasheet, and equal to $4x10^8$ beads ml^{-1}. Average differentiation with ~20% CD31+ ECs will give ~$2X10^5$ ECs per 6-well. Therefore we propose adding ~ 7 µl beads per 6-well (or ~ $2\text{-}3x10^6$ beads per 6-well) i.e. on average ~10 beads per cell.

18| Wash beads 2x with 1 ml DMEM-0.1%BSA by pipetting them into 5-ml FACS tube and placing the tube into small DynaMag™-5 magnet. Aspirate medium with a glass Pasteur pipette and perform a second wash as stated above.

19| Resuspend beads in ~3 times excess volume (~21 µl) of the initial volume of beads in DMEM-0.1% BSA.

20| Aspirate differentiation medium from the cells from step 15 and wash cells once with PBS (without Ca^{2+}/Mg^{2+}). Add 2ml of DMEM-0.1% BSA to the cells and pipet ~21µl of beads directly to the cells with gentle agitation.

21| Seal the 6-well plates with Parafilm and place the plates on the rotating plate machine with gentle agitation (10 rpm).
22| Incubate cells with beads for 20-30 min at RT. ECs can be identified upon binding of magnetic beads to the cell surface and appearance of a brownish precipitate that also becomes visible by eye.
? TROUBLESHOOTING

23| Aspirate DMEM-0.1% BSA and wash cells once with PBS (without Ca^{2+}/Mg^{2+}).
24| Aspirate PBS and add 0.5ml 1x TrypLE per well of the 6-well plate. Incubate cells with TrypLE for 5-8 min at RT.
25| Stop the reaction by adding 2 ml FACSB-10 per well.
26| Place a CellTrics filter on top of a 5-ml FACS tube, or 15-ml conical tube depending on the total volume. Apply the cell suspension from step 25| and wash the filter additionally with 1-2ml of FACSB-10.
▲ **CRITICAL STEP** DynaMag™-5 for 5ml tube is used for the isolation of cells from one 6-well, and DynaMag™-15 for 15ml tube is recommended for isolation of cells from one 6-well plate.
27| Place cells on the magnet (DynaMag™-5 or DynaMag™-15). Collect the supernatant (CD31- fraction) into a separate tube, put the CD31- fraction aside and keep it at RT until the end of the isolation. The CD31- fraction will be used to derive pericytes; see step 30|, option B.
28| Remove the tube with the beads from the magnet and resuspend the beads in 2ml (5-ml tube) or 5ml (15-ml tube) of the FACSB. Place the tube back on the magnet and aspirate the supernatant. Repeat the washing step once more with FACSB and twice more with DMEM-0.1% BSA.
29| Retain the beads with cells bound, proceed to step 30 option A to use this CD31+ fraction to derive ECs.

Expansion of isolated cells

30| Expand the appropriate fraction from the earlier section to either ECs (option A) or pericytes (option B). The CD31+ cell fraction from the Dynabead isolation (from Step 29|) are expanded to ECs and the CD31- cell fraction (from Step 27|) are used to establish endothelial and pericyte cell cultures.

(A) Expansion of ECs •TIMING 4 days

(i) Resuspend cells from step 29| in 2ml of EC-SFM (FULL).
(ii) Coat a T75 tissue culture flask with gelatin as described in EQUIPMENT SETUP. Aspirate gelatin and add EC-SFM (FULL) to the T75 cell culture flask.
(iii) Count the total number of CD31+ ECs, and plate approximately 5000cells/cm^2 or we routinely plate all the CD31+ ECs derived from one full 6-well plate into one T75 tissue culture flask.
(iv) Incubate cells until they reach confluence, at which point proceed to step 31.

(B) Expansion of pericytes •TIMING 10 days

(i) Centrifuge the CD31- fraction of cells from step 27| at 300g for 3 min.

(ii) Coat a T75 tissue culture flask with gelatin as described in EQUIPMENT SETUP. Aspirate gelatin and add EGM-2 to the T75 cell culture flask.

(iii) Aspirate the supernatant from the cells and resuspend the cell pellet in 4ml of EGM-2.

(iv) Count the total cell yield and plate approximately 10000 cells/cm^2 on the gelatin-coated flasks. Incubate for 3-4 days until cells reach ~80% confluence.

(v) Passage cells with TrypLE in a one to three dilution in DMEM-10% FBS with 2ng/ml TGFβ3 and 4ng/ml PDGF-BB and incubate 3 days.

(vi) Replace medium on cells with DMEM-10% FBS.

(vii) Expand cells until they reach 80-90% confluence and then proceed to step 31.

Characterization and cryopreservation of expanded ECs and pericytes

31| After isolation, a subset of the ECs and Pericytes can be characterized by flow cytometry (option A) or immunocytochemical staining (option B) or cryopreserved (option C). Flow cytometry can also be used on cells prior to isolation with CD31-Dynabeads (from step 15) to estimate monolayer differentiation efficiency.

(A) Flow cytometric analysis of isolated ECs and Pericytes •TIMING 3h.

(i) Aspirate differentiation medium and wash cells once with PBS (without Ca^{2+}/Mg^{2+}).

(ii) Aspirate PBS and add 0.5ml 1x TrypLE per 6-well. Incubate cells with TrypLE for 5-8 min at RT.

(iii) Stop reaction by adding 2 ml per well FACSB-10.

(iv) Place a CellTrics filter on top of a 5-ml FACS tube. Apply cell suspension from previous step and wash the filter additionally with 1-2ml of FACSB-10.

(v) Spin down the cell suspension at 300g for 3 min.

(vi) Wash the cell suspension once by resuspending in 1 ml FACSB and spinning down at 300g for 3 min.

(vii) Incubate aliquots of the cell suspension with mixes of antibodies in the dark for 45 min at 4°C. Perform immunocytochemical staining with the following antibodies: anti-VE-cadherin-A488, CD31-APC and PDGFRβ-PE and KDR-PE at concentrations listed in the reagents section.

▲ **CRITICAL STEP** Depending on the flow cytometer's laser setup, the user might consider preparing "compensation samples", such as single stained cells. The user should consider discussing setup of the flow cytometer experiment with an experienced FACS operator. Include appropriate controls, such as non-stained cells, as well as isotype matches, i.e. antibodies generated in the same species and the same isotype conjugated to the fluorochrome used in the actual experiment (mouse-anti-human-IgG1-A488, anti-human-IgG1-APC, anti-human-IgG1-PE).

(viii) Wash the cell suspension once by resuspending in 1 ml FACSB and spinning down at 300g for 3 min.

PAUSE POINT Flow cytometer analysis can be performed either immediately or the cell suspension can be post-fixed with 1% PFA and analyzed the next day.

(ix) Perform analysis with a flow cytometer. For the flow cytometric analysis here, the MACSQuant VYB (Miltenyi) with the following instrument settings was used: Blue/488 FITC, A488: 525/50; Yellow/561 PE: 586/15, APC: 661/20, APC-Cy7: 750LP. FlowJo software was used for data analysis.

▲ **CRITICAL STEP** Routinely, the laser setup commonly found on the LSRII (BD) flowcytometer will require the following laser settings: Blue/488 FITC, A488: 505LP-530/30; Yellow/561 PE: 570LP-582/15, APC: 635LP-660/20. For the MACSQuant VYB (Miltenyi) flowcytometer, the following laser settings will be required: Blue/488 FITC, A488: 525/50; Yellow/561 PE: 586/15, APC: 661/20. MACSQuant laser setup might require additional compensation between PE and APC channels.

▲ **CRITICAL STEP** Should the user wish to sort cells using FACS, the standard Aria SORP setup with the pressure and nozzle combination 45 PSI / 85 µm is advisable.

(B) Immunocytochemical staining of isolated ECs and Pericytes •TIMING 2 days.

(i) Plate cells on fibronectin- (for ECs) or gelatin- (for pericytes) coated glass coverslips. Grow ECs in EC-SFM (FULL) medium until they form a confluent monolayer (approximately 3-4 days). Grow pericytes in DMEM-10% FBS together with TGFβ3 (2ng/ml) and PDGF-BB (4ng/ml) for 72h.

(ii) Aspirate cell growth medium and fix cells with 4%PFA (vol/vol) for 10 min at RT. Wash three times with PBS for 5 min per each wash.

■ **PAUSE POINT** Samples can be stored in PBS for up to one week at 4°C.

(iii) Permeabilize with cells by placing in 0.5 % TX-100 in PBS (vol/vol) for 3 min at RT.

(iv) Block cells with 1%BSA (wt/vol)/2% goat serum (vol/vol) in PBS for 1h at RT.

(v) Add primary antibodies against endothelial and pericyte specific markers at concentrations listed in the reagents section in 1%BSA (wt/vol) in PBS to appropriate coverslips. Keep non-stained coverslips with cells for negative control. Incubate overnight at 4 °C.

(vi) Wash three times with PBS for 10 min per each wash.

(vii) Add secondary antibodies (1:300) in 1%BSA (wt/vol) in PBS. Use secondary antibody addition only without primary antibodies as a negative control. Incubate 1h at RT.

(viii) Wash three times with PBS for 20-30 min per wash.

(ix) Add DAPI (1:1000 in PBS) for 10 min.

(x) Mount coverslips on cover glass with a drop of fluorescence mounting medium (DAKO). Dry slides overnight at RT.

■ **PAUSE POINT** Slides can be stored, protected from light, at RT for up to one year.

(C) Cryopreservation of Isolated ECs and Pericytes •TIMING 2h for cryopreservation and 4-5d for thawing

(i) When cells have been characterized, prepare cryopreservation medium (40% EGM-2, 10% DMSO, 50% FBS)vol/vol)), filter sterile and pre-chill by placing it on ice.

(ii) Aspirate the EC growth medium from the cells growing in steps 30Aiv or 30Bvii wash cells once with PBS and add TrypLE. Incubate cells with TrypLE for 5min at RT. Stop the reaction with a double volume of EGM-2. Centrifuge cells at 300g for 3 min.

(iii) Aspirate supernatant and gently resuspend the cell pellet in the cryopreservation medium. Freeze down 25cm^2 of ECs and 37.5cm^2 of pericytes per cryovial.

(iv) Transfer cryovials to -80°C. After a minimum of 3 days, transfer the cryovials into liquid N_2 for prolonged storage.

■ **PAUSE POINT** Cryopreserved cells can be stored for an unlimited period in liquid N_2.

(v) Thaw the cryopreserved ECs and pericytes in a water bath at 37°C.

(vi) Transfer cells into a 15-ml falcon tube containing 10ml of DMEM-10% (for pericytes) or EC-SFM (for ECs).

(vii) Centrifuge cells at 300g for 3min. Aspirate the supernatant, gently resuspend the pellet and transfer it into a flask containing EC growth medium. One cryovial of ECs (25cm^2) can be started up in one T75, and one cryovial of pericytes (37.5cm^2) can be started up in one T75. These cells can be routinely used in functional assays 4-5 days after thawing.

(viii) Cells can be further expanded by passaging. ECs are passaged 1:3 upon reaching confluence and can be propagated up to passage 5. Pericytes can be passaged 1:3 every 5 days and propagated up to passage 10.

Functional competence of ECs and Pericytes derived from hPSCs

32| In order to test the functionality of the isolated ECs and Pericytes, a co-culture assay with both cell types can be performed (option A). In addition, EC functionality can be tested in a zebrafish xenograft (option B).

(A) Establishing an *in vitro* model: co-culture of EC with pericytes and smooth muscle cells •TIMING 10 days

(i) Prepare gelatin coated 96-well plates (use black imaging plates).

(ii) Aspirate cell growth medium from flasks containing characterized ECs and pericytes (one confluent T-75 of ECs and two to three T-75 flask of pericytes at 80% confluency, from steps 31Cviii are routinely needed for one 96-well plate) and wash cells once with PBS. Add 4 ml TrypLE and incubate at RT for 3-5 min (ECs) and 5-8 min (pericytes).

(iii) Add a double volume of EGM-2 to the cells and transfer them into a 15-ml conical tube.

(iv) Centrifuge cells at 300g for 3min.

(v) Resuspend pericytes in 10ml of EGM-2 medium and count total cell yield. Keep cells aside.

(vi) Wash ECs with EBM-2 (4ml), count the total yield of ECs.

(vii) ***General cytoplasmic labeling of ECs:*** Resuspend EC cells with 1ml Diluent C (provided as a part of PKH-67 labeling kit, or can be purchased separately from Sigma)(A). In a separate tube mix 1ml of Diluent C with 4µl of PKH-67 (B), tap gently with the finger. Add 1ml of B to A, tap gently with the finger and incubate for 5 min at RT protected from light. Stop the reaction with 2ml

EGM-2. Centrifuge cells at 300g for 3 min. Wash additionally once with 2ml EGM-2.

▲ **CRITICAL STEP** General cytoplasmic labeling provided for one T-75 flask of confluent ECs what is ~1.5-2X10^6 cells. In case of more cells the conditions and amount of the PKH-67 labeling agent should be adjusted accordingly.

(viii) Calculate the total number of ECs and pericytes needed for the assay based on the number of wells you wish to have. Each well needs to be seeded with 12x10^3 ECs plus 50X10^3 P (i.e. a ratio of 12EC:50P).

(ix) Mix the appropriate number of EC and P cells in a 15-ml tube by gently inverting the tube 3-4 times.

(x) Centrifuge cells at 300 g for 3 min.

(xi) Resuspend cells with the appropriate volume of EGM-2. Use 200µl EGM-2 per well.

(xii) Transfer 200µl of EGM-2 per well to a 96-well plate with a dispenser.

(xiii) Centrifuge the plate for 1 minute at 300 g. Place the plate back to the cell culture incubator.

(xiv) Replace EGM-2 medium on day 1 and day 4. Optionally, test compounds can be added to the EGM-2 medium on day 1 and day 4, as described in our recent publication[21]. We move onto step xv when cells have been co-cultured for 7 days.

? **TROUBLESHOOTING**

(xv) ***Immunocytochemical staining of the co-culture:*** Aspirate the EC growth medium and add 100 µl of the 4% PFA in PBA (vol/vol). Incubate for 15 min at RT. Aspirate PFA and wash the co-culture three times with 200 µl of PBS for 5 min for each wash.

(xvi) Permeabilize cells with 200 µl of 0.5 % TX-100 in PBS (vol/vol) for 5 min.

(xvii) Block with 200 µl of 1% BSA (wt/vol)/0.1% TX-100 (vol/vol)/2% goat serum (vol/vol) in PBS for 1h at RT.

(xviii) Add 100 µl of primary anti-CD31 antibodies (1:200) in 1%BSA (wt/vol)/0.1% TX-100 in PBS. Incubate overnight at 4 °C.

(xix) Wash twice with 200 µl of 0.1% TX-100 and once more with 200 µl of PBS with gentle agitation for 20-30 min per wash.

(xx) Add 100 µl of appropriate secondary antibodies (1:300) in 1% BSA (wt/vol) in PBS. Incubate 1h at RT in dark.

(xxi) Wash once with 200 µl of PBS for 20-30 min under gentle agitation.

■ **PAUSE POINT** Samples can be stored in PBS for up to 6 months at 4°C**.** For the co-staining (see step xxiv) it is better to process as soon as possible.

(xxii) ***Image acquisition of the co-culture:*** Image the co-culture with the automatic imaging system (example BD Pathway 855) with a 4x objective. Acquire the area covered with a 2x2 montage. If you wish to co-stain with anti-SM22 or Ki67, proceed with step xxiv.

(xxiii) ***Quantification:*** Quantify the co-culture with the AngioTool software[41]. This determines the "area occupied" (equivalent to the amount of organized vasculature) and the number of branch points in the vasculature.

(xxiv) ***Co-staining with anti-SM22 or Ki67 (Optional):*** Add 100 µl of primary anti-SM22 (1:300) or Ki67 antibodies (1:50) in 1%BSA (wt/vol) in PBS. Incubate overnight at 4 °C.

(xxv) Wash three times with 200 µl PBS for 10 min per wash.

(xxvi) Add secondary antibodies (1:300) in 1% BSA (wt/vol) in PBS. Incubate 1h at RT.

(xxvii) Wash three times with 200 µl PBS, 20-30min per wash.

(xxviii) Add DAPI (1:1000 in 100 µl PBS) for 10 min.

(xxix) Aspirate DAPI; add 200 µl PBS.

■ **PAUSE POINT** Samples can be stored in PBS for up to 6 months at 4°C.

(xxx) ***Image acquisition of the co-culture:*** Image the co-culture with the automatic imaging system (example BD Pathway 855) with a 10x objective. Acquire the area covered with a 3x3 montage. For better resolution images can be acquired with the confocal microscope with a 20x objective.

(xxxi) ***Quantification:*** Quantify images: we quantify the area covered by SM22 positive pericytes, or the number of Ki67 positive ECs using a custom pipeline with CellProfiler[42].

(B) Establishing *in vivo* model: integration of EC into zebrafish (48 hpf)

•TIMING 10days

CRITICAL For detailed description on zebrafish work please see http://zfin.org/zf_info/zfbook/zfbk.html or "The Zebrafish Book" by Westerfield [55].

(i) Set up breeding of an adult Tg(fli1:GFP) zebrafish prior to the end of the light cycle.

Zebrafish will initiate laying and fertilization of eggs at the end of next light cycle.

(ii) Collect fertilized eggs and keep at 28°C.

▲ **CRITICAL STEP** There are many methods for the preparation and collection of fertilized eggs. Tanks can be lined with fine netting, so that the eggs fall through the fine fabric and thus escape predation. Marbles can also be used to cover the bottom of the tank. Discuss which method is used at your zebrafish facility.

(iii) At ~6 hpf (hours past fertilization), separate live embryos from unfertilized eggs, dead or badly formed embryos by light microscopy. Retain live embryos.

(iv) Distribute approximately 60 embryos per Petri dish filled with egg water and keep at 28°C for a further 36 h.

(v) Separate live embryos from dead or badly formed embryos once more (this is at ~24 hpf) by light microscopy.

(vi) Separate GFP (Tg(fli1:GFP)) expressing embryos from negative embryos by fluorescent microscopy.

(vii) Refresh egg water, and return Petri dish to the incubator at 28°C for 22 h.

(viii) ***Embryo dechorionation:*** Dechorionate any embryos still surrounded by the chorion. To do this, use the fine tips of one of the forceps, carefully gripping the membrane without harming the embryo (**Fig. 6a,b,c**). With the other set of forceps, carefully grip the shell and pull the shell in opposite directions. The shell will tear open and the embryo will swim free. Approximately 50-100 embryos are needed per test condition, thus dechrionate enough for

sufficient embryos to be available. Return dechorionated embryos to the 28°C incubator until the cells have been prepared for injection (step x).

▲ **CRITICAL STEP** ~46 hpf embryos should remain in the egg water throughout the dechorionation process.

▲ **CRITICAL STEP** Around ~48 hpf some zebrafish embryos can be seen swimming freely without the chorion, since the hatching phase starts around 48-72 hpf.

(ix) ***Cell preparation:*** Aspirate cell growth medium from one T25 flask with ECs at 80-90% confluence and wash cells once with PBS. Add 1 ml TrypLE and incubate at RT for 3-5 min. Label cells with Cm DiI according to the manufacturer's instructions. Count cell number. Resuspend cells in an appropriate volume of EBM-2 medium. Place the suspension of cells and EBM-2 medium on ice.

▲ **CRITICAL STEP** The concentration of cells for injections should be optimized by the researcher, as this is highly dependent on many intangible variables such as the user's ability, the size of the needle opening, room humidity, etc. It is best to start with a highly concentrated suspension (for example, start with 2.8 x 10^6 in 50 µl), where medium can be added until the right concentration can be determined by the researcher

(x) **Place the suspension of fluorescent cells at RT for approximately 5 min prior to implantation, during this incubation proceed with step xi.**

(xi) ***Implantation procedure:*** Anaesthetize dechorionized zebrafish embryos with 1X of Tricaine diluted in egg water in a Petri dish kept at RT.

(xii) Collect five to ten aestheticized embryos with a plastic transfer pipette and position on a 10cm Petri dish coated with 3% agarose.

▲ **CRITICAL STEP** Excess water surrounding the embryos can be removed using a tissue. It is important to keep the embryos moist; however too much moisture is detrimental to the injection procedure. It is up to the researcher to find the right balance of moisture.

▲ **CRITICAL STEP** It is best to inject the embryos in groups of ten, as this provides a number that is easily counted and enough embryos that can be processed quickly without the agarose plate drying out.

(xiii) Resuspend the cell suspension from step xi by repeatedly pipetting with a 200 µl Pipette tip in order to break up any clumps of cells.

(xiv) Break the tip of the borosilicate glass capillary needles with the stainless steel forceps.

▲ **CRITICAL STEP** Note that the needle opening must be large enough for the cells to be implanted without damage, but thin enough so as not to cause harm to the zebrafish. The size of the needle opening must be optimized by the user.

(xv) Load up to 20 µl of the single cell suspension into the borosilicate glass capillary needles using a 20 µl pipette and microloader pipette tips.

(xvi) Optimize the Pneumatic PicoPump injection by injecting cells onto the agarose plate.

(xvii) Estimate the number of injected cells by the size of the circular droplet. Approximately 400 cells are required per embryo.

▲ **CRITICAL STEP** The pressure and time limit can be altered until the correct number of cells is achieved.

▲ **CRITICAL STEP** Note that it is better to alter the timing of the injections rather than the pressure; zebrafish embryos cannot tolerate very high pressure.

(xviii) Perform implantation using a Pneumatic Picopump and use a manipulator as recommended by manufacturer's instructions.

(xix) Inject the cells approximately 60µm above the ventral end of the duct of Cuvier (DoC), where the DoC opens into the heart (**Fig. 6d**).

▲ **CRITICAL STEP** It is important to recalibrate the number of cells per injection throughout the assay. Due to gravity, the cells in the suspension will settle towards the tip of the needle, and thus alter the number of cells per injection.

▲ **CRITICAL STEP** Visualize the duct of Cuvier as a wide stream of blood cells entering the heart (blue dashed line)(**Fig. 6d**). Inject ECs above the point that the duct of Cuvier enters the heart (red circle and arrow)(**Fig. 6d**).

(xx) Remove the embryos from the agarose plate and return them to the fresh egg water with the plastic transfer pipette.

▲ **CRITICAL STEP** The zebrafish should recover from the Tricaine within a minute.

▲ **CRITICAL STEP** Too much Tricaine is detrimental to the survival of the fish. Ensure that a 1x working dilution of Tricaine is used.

(xxi) On the same day of injection examine whether cells are present in the circulation using fluorescent microscopy.

(xxii) Maintain the correctly implanted embryos at 33°C

(xxiii) Keep embryos in a Petri dish, smaller 6-well plates, or place individual fish into a well of a 96-well plate. Supply plentiful egg water.
Refresh egg water at least every second day. Monitor embryo survival rate every day.

▲ **CRITICAL STEP** Experiments should be discarded when the survival rate of the control group is less than 80% over the 6 days.

(xxiv) ***Analysis on live embryos:*** analyze live embryos with a stereo fluorescent microscope at any time throughout the experiment.

▲ **CRITICAL STEP** It is best to keep the embryos in egg water and disturb them as little as possible.

(xxv) ***Fixation of zebrafish embryos:*** At day 5 post implantation, fix the embryos by overnight incubation in 4% PFA at 4˚C.

■ **PAUSE POINT** Samples can be stored in PBS for up to 6 months at 4°C.

(xxvi) Analyse the fixed embryos using, for example, confocal microscopy or immunohistochemistry.

? TROUBLESHOOTING

Troubleshooting guidance can be found in Table 1.

Step	Problem	Possible cause of the Problem	Solution to solve the Problem
4	hPSC colonies do not detach	Old dispase solution	Prepare fresh dispase solution
10	hPSC pieces are too small	hPSCs break up easily	Use less force to break up hPSCs into pieces
15	No ECs can be seen at day 10	(a) Differentiation did not work (b) The cell density is too high and ECs are difficult to identify	(a) Check growth factor concentrations, perform an additional titration step for BMP4 and ActivinA (b) Perform flow cytometry analysis prior to isolation with CD31-Dynabeads
22	No attached CD31-Dynabeads can be observed	(a) Old Dynabeads (b) Differentiation did not work	(a) Check whether beads bind to control HUVEC or another type of ECs. (b) Perform flow cytometric analysis prior to isolation with CD31-Dynabeads
32|xiv	Endothelial cell sprouts do not develop at day 4	(a) Old EGM-2 medium (b) High pericyte passage (above passage 10)	(a) Prepare fresh EGM-2 medium (b) Start up low passage pericytes

•TIMING

See Figure 1.

ANTICIPATED RESULTS

The differentiation process can be monitored as morphological/phenotypic changes in the culture. Firstly, the appearance of mesenchymal cells that migrate out from the hPSCs colonies can be observed on day 3-4 of differentiation (**Fig. 2f,g**). EC islands appear around day 6-7 of differentiation (**Fig. 2,ij**). In addition, the differentiation efficiency can be controlled by flow cytometry. Routinely, 20-30% of VE-cadherin/CD31+ cells can be expected during differentiation at day 7-10 (**Fig. 3**). The earliest VE-cadherin appears around day 5-7 and at day 10, most VE-cadherin+ cells express CD31.

Freshly isolated ECs are highly proliferative. They normally reach confluence 2-3 days after isolation (**Fig. 4a**). Routinely we achieve 98% EC purity after one round of selection; this can be observed morphologically (**Fig. 4a,c**) or by flow cytometric analysis[21]**.** The expansion of the CD31- fraction of cells results in the establishment of a homogenous population of pericytes (**Fig. 4b,d**); these are negative for EC markers and express PDGFRβ in addition to other pericyte markers[21].

On average, from two 6-well plates of initiating differentiation cultures or two wells of a 6-well plate of hPSC culture, we can routinely derive and freeze down up to 2-3x10^6 ECs at passage 1, and 3-4x10^6 pericytes at passage 3. Cells can be efficiently cryopreserved with a viability >90% without affecting their functionality. ECs should be passaged upon reaching 90-100% confluence at an average ratio of 1:3 (at passage 2 and passage 3) and 1:2 (at passage 4). They can be propagated further for an additional 3-4 passages until passage 5. However, a slight decrease in proliferation rate may occur after passage 5. Pericytes should be passaged upon reaching ~80% confluence on average 1:3. Pericytes can be propagated until passage 15 although their ability to support EC-sprouting tends to decrease after passage 10.

Co-culture:

Upon co-culture ECs readily remodel and form well-organized networks on top of the monolayer of pericytes. ECs can be visualized by green fluorescence (in the case of general membrane labeling). We terminate the assay on day 7. ECs are additionally visualized with the anti-CD31 antibody **(Fig. 5a)**, as this makes quantification easier, since general cytoplasmic dyes tend to give patchy staining. Pericytes recruited to the EC sprouts can be visualized with the anti-SM22 maker **(Fig. 5c)**. Proliferation can be assessed upon visualization with the Ki67 marker. Quantification of the endothelial network can be done with freely available software AngioTool **(Fig. 5b)**[41]. The area covered by SM22 positive cells or Ki67 positive proliferative ECs can also be quantified easily with custom pipeline based on CellProfiler **(Fig. 5d)**[21,42].

Zebrafish:

Within the first few hours post transplantation, red fluorescent ECs will be circulating within the zebrafish's circulatory loop that can be visualized by GFP expressed in the zebrafish vasculature. Note, many cells may still be located within the yolk sac - it is almost impossible to inject all cells into the embryonic circulation. After 24 hours, the fluorescent signal from the transplanted cells may fade and disappear completely in many of the zebrafish. This loss of fluorescence in zebrafish is similar to the loss of bioluminescence seen in human carcinoma cells injected into nude mice, described by a 2004 study by Rosol and colleagues[56]. However, as the cells begin to recover and survive in the new microenvironment, bright red fluorescent signal can be observed along the dorsal aorta and caudal vein (**Fig. 7a**). In some cases, cells can also incorporate into intersegmental vessels or small vessels in the heart and brain (**Fig. 7b,c,d**). hPSC-derived ECs were able to integrate into embryonic vasculature of the zebrafish. Approximately ~80% of injected zebrafish showed the presence of hPSC-derived ECs at day 5 post implantation, whereas only ~20% of zebrafish demonstrated the presence of HUVECs[21].

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Author Contributions V.V.O. designed the protocol, supervised the project, wrote the manuscript, established the monolayer differentiation; isolation and expansion of ECs and pericytes, co-culture experiment and performed all of the cell experiments. F.E.H. performed all of the cell experiments and wrote part of the protocol. S.P.R. performed characterization of the monolayer differentiation, the co-culture experiments and developed the pipeline for the quantification of the vascular sprouts with the cells CellProfiler. Y.D. performed zebrafish xenotransplantation experiments and wrote the zebrafish protocol. P.D. supervised the project and edited the manuscript. C.L.M. designed the protocol, supervised the project and wrote the manuscript.

Competing financial interests The authors declare that they have no competing financial interests.

REFERENCES

1. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147 (1998).
2. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131,** 861–872 (2007).
3. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318,** 1917–1920 (2007).
4. Chambers, S. M. *et al.* Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat. Biotechnol.* **27,** 275–280 (2009).
5. Touboul, T. *et al.* Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* **51,** 1754–1765 (2010).
6. Green, M. D. *et al.* Generation of anterior foregut endoderm from human embryonic and induced pluripotent stem cells. *Nat. Biotechnol.* **29,** 267–272 (2011).
7. Spence, J. R. *et al.* Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* **470,** 105–109 (2011).
8. Elliott, D. A. *et al.* NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat. Methods* **8**, 1037–1040 (2011). doi:10.1038/nmeth.1740
9. Dubois, N. C. *et al.* SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nat. Biotechnol.* **29,** 1011–1018 (2011).
10. Huang, S. X. L. *et al.* efficient generation of lung and airway epithelial cells from human pluripotent stem cells. *Nat. Biotechnol.* 1–11 (2013). doi:10.1038/nbt.2754
11. Lancaster, M. A. *et al.* Cerebral organoids model human brain development and microcephaly. *Nature* **501,** 373–379 (2013).
12. Ingram, D. A. *et al.* Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **104,** 2752–2760 (2004).
13. Yoder, M. C. *et al.* Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* **109,** 1801–1809 (2007).
14. Armulik, A., Genové, G. & Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* **21,** 193–215 (2011).
15. Winkler, E. A., Bell, R. D. & Zlokovic, B. V. Central nervous system pericytes in health and disease. *Nature Neuroscience* **14,** 1398–1405 (2011).
16. Lebrin, F. *et al.* Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. *Nat. Med.* **16,** 420–428 (2010).
17. Chintalgattu, V. *et al.* Coronary microvascular pericytes are the cellular target of sunitinib malate-induced cardiotoxicity. *Sci Transl Med* **5,** 187ra69 (2013).
18. Crisan, M. *et al.* A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. *Cell Stem Cell* **3,** 301–313 (2008).
19. Bianco, P. *et al.* The meaning, the sense and the significance: translating the

science of mesenchymal stem cells into medicine. *Nat. Med.* **19,** 35–42 (2013).
20. Frenette, P. S., Pinho, S., Lucas, D. & Scheiermann, C. Mesenchymal Stem Cell: Keystone of the Hematopoietic Stem Cell Niche and a Stepping-Stone for Regenerative Medicine. *Annu. Rev. Immunol.* **31,** 285–316 (2013).
21. Orlova, V. V. *et al.* Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler. Thromb. Vasc. Biol.* **34,** 177–186 (2014).
22. Levenberg, S. Endothelial cells derived from human embryonic stem cells. *Proceedings of the National Academy of Sciences* **99,** 4391–4396 (2002).
23. Dar, A. *et al.* Multipotent Vasculogenic Pericytes From Human Pluripotent Stem Cells Promote Recovery of Murine Ischemic Limb. *Circulation* **125,** 87–99 (2012).
24. Choi, K.-D. *et al.* Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells* **27,** 559–567 (2009).
25. Nourse, M. B. *et al.* VEGF Induces Differentiation of Functional Endothelium From Human Embryonic Stem Cells: Implications for Tissue Engineering. *Arterioscler. Thromb. Vasc. Biol.* **30,** 80–89 (2009).
26. Adams, W. J. *et al.* Stem Cell Reports. *Stem Cell Reports* **1,** 105–113 (2013).
27. Vodyanik, M. A. *et al.* A Mesoderm-Derived Precursor for Mesenchymal Stem and Endothelial Cells. *Cell Stem Cell* **7,** 718–729 (2010).
28. Kusuma, S. *et al.* Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. *Proceedings of the National Academy of Sciences* 110, 12601–12606 (2013). doi:10.1073/pnas.1306562110
29. James, D. *et al.* Expansion and maintenance of human embryonic stem cell-derived endothelial cells by TGFbeta inhibition is Id1 dependent. *Nat. Biotechnol.* **28,** 161–166 (2010).
30. Samuel, R. *et al.* Generation of functionally competent and durable engineered blood vessels from human induced pluripotent stem cells. *Proceedings of the National Academy of Sciences* **110,** 12774–12779 (2013).
31. Park, T. S. *et al.* Vascular progenitors from cord blood-derived induced pluripotent stem cells possess augmented capacity for regenerating ischemic retinal vasculature. *Circulation* **129,** 359–372 (2014).
32. White, M. P. *et al.* Limited Gene Expression Variation in Human Embryonic Stem Cell and Induced Pluripotent Stem Cell-Derived Endothelial Cells. *Stem Cells* **31,** 92–103 (2012).
33. Costa, M. *et al.* Derivation of endothelial cells from human embryonic stem cells in fully defined medium enables identification of lysophosphatidic acid and platelet activating factor as regulators of eNOS localization. *Stem Cell Res* **10,** 103–117 (2013).
34. Lippmann, E. S. *et al.* Derivation of blood-brain barrier endothelial cells from human pluripotent stem cells. *Nat. Biotechnol.* **30,** 783–791 (2012).
35. Cheung, C., Bernardo, A. S., Trotter, M. W. B., Pedersen, R. A. & Sinha, S. Generation of human vascular smooth muscle subtypes provides insight into embryological origin–dependent disease susceptibility. *Nat. Biotechnol.* **30,** 165–173 (2012).
36. Ng, E. S., Davis, R., Stanley, E. G. & Elefanty, A. G. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nat Protoc* **3,** 768–776 (2008).

37. Lian, X. *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences* **109,** E1848–57 (2012).
38. Evensen, L., Micklem, D. R., Link, W. & Lorens, J. B. A novel imaging-based high-throughput screening approach to anti-angiogenic drug discovery. *Cytometry A* **77,** 41–51 (2010).
39. Evensen, L., Link, W. & Lorens, J. B. Image-based high-throughput screening for inhibitors of angiogenesis. *Methods Mol. Biol.* **931,** 139–151 (2013).
40. Evensen, L. *et al.* Mural cell associated VEGF is required for organotypic vessel formation. *PLoS ONE* **4,** e5798 (2009).
41. Zudaire, E., Gambardella, L., Kurcz, C. & Vermeren, S. A Computational Tool for Quantitative Analysis of Vascular Networks. *PLoS ONE* **6,** e27385 (2011).
42. Jones, T. R. *et al.* CellProfiler Analyst: data exploration and analysis software for complex image-based screens. *BMC Bioinformatics* **9,** 482 (2008).
43. Stoletov, K. & Klemke, R. Catch of the day: zebrafish as a human cancer model. *Oncogene* **27,** 4509–4520 (2008).
44. Goessling, W., North, T. E. & Zon, L. I. New waves of discovery: modeling cancer in zebrafish. *Journal of Clinical Oncology* **25,** 2473–2479 (2007).
45. Zon, L. I. & Peterson, R. T. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* **4,** 35–44 (2005).
46. Isogai, S., Lawson, N. D., Torrealday, S., Horiguchi, M. & Weinstein, B. M. Angiogenic network formation in the developing vertebrate trunk. *Development* **130,** 5281–5290 (2003).
47. Naber, H. P. H., Drabsch, Y., Snaar-Jagalska, B. E., Dijke, ten, P. & van Laar, T. Snail and Slug, key regulators of TGF-β-induced EMT, are sufficient for the induction of single-cell invasion. *Biochem. Biophys. Res. Commun.* **435,** 58–63 (2013).
48. Zhang, L. *et al.* TRAF4 promotes TGF-β receptor signaling and drives breast cancer metastasis. *Mol. Cell* **51,** 559–572 (2013).
49. Zhang, L. *et al.* USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor. *Nat. Cell Biol.* **14,** 717–726 (2012).
50. He, S. *et al.* Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model. *J. Pathol.* **227,** 431–445 (2012).
51. Ghotra, V. P. S. *et al.* Automated whole animal bio-imaging assay for human cancer dissemination. *PLoS ONE* **7,** e31281 (2012).
52. van de Stolpe, A. *et al.* Human embryonic stem cells: towards therapies for cardiac disease. Derivation of a Dutch human embryonic stem cell line. *Reproductive BioMedicine Online* **11,** 476–485 (2005).
53. Dambrot, C. *et al.* Polycistronic lentivirus induced pluripotent stem cells from skin biopsies after long term storage, blood outgrowth endothelial cells and cells from milk teeth. *Differentiation* **85,** 101–109 (2013).
54. Davis, R. P. *et al.* A protocol for removal of antibiotic resistance cassettes from human embryonic stem cells genetically modified by homologous recombination or transgenesis. *Nat Protoc* **3,** 1550–1558 (2008).
55. Westerfield, M. *The Zebrafish Book*. (2007).
56. Rosol, T. J., Tannehill-Gregg, S. H., Corn, S., Schneider, A. & McCauley, L. K. Animal models of bone metastasis. *Cancer Treat. Res.* **118,** 47–81 (2004).

FIGURE LEGENDS

Figure 1 Timeline of the protocol procedures.

Figure 2 Phase contrast images of hPSCs at different stages of differentiation. (a) hPSC colony prior to passaging, (b) representative piece of the colony after passaging, (c) small colony the day after passaging, (d) approximate size of the colony at the time point of induction of differentiation, (e,f) morphological changes upon mesoderm induction and (g,h,i,j,k,l) vascular specification. (f,g) Emergence of mesenchymal cells can be observed on day 3-4 of differentiation, and (i,j) first islands of ECs appear between day 6-7 of differentiation; (k,l) these are further expanded till day 9-10. Scale bar 500 μm.

Figure 3 Flow cytometric analysis of VE-cadherin and CD31 expression at different time points of differentiation. (a) Representative non-stained, and single stained (VE-cadherin-A488 and CD31-APC) samples at day 10 of differentiation, gated from the live cell population (P1). (b) Representative time course showing the live cell population and appearance of VE-cadherin/CD31 positive cells within the live gate (P1).

Figure 4 Characterization of isolated ECs and pericytes. (a,b) Phase contrast image of isolated ECs and pericytes . (c) Representative confocal images of CD31, VE-cadherin, vWF and SMA immunocytochemical staining (in green) and DAPI (in blue) in hPSC-derived ECs . (d) Representative confocal images of SM22 (in white), Caldesmon (in red), SMA (in green) and DAPI (in blue), and overlay of immunocytochemical staining in hPSC-derived pericytes upon culture for 3 days in EGM-2, DMEM-10% FBS and DMEM-10% FBS with additional TGFβ and PDGF-BB. Scale bar (a,b) 500 μm, (c,d) 100 μm.

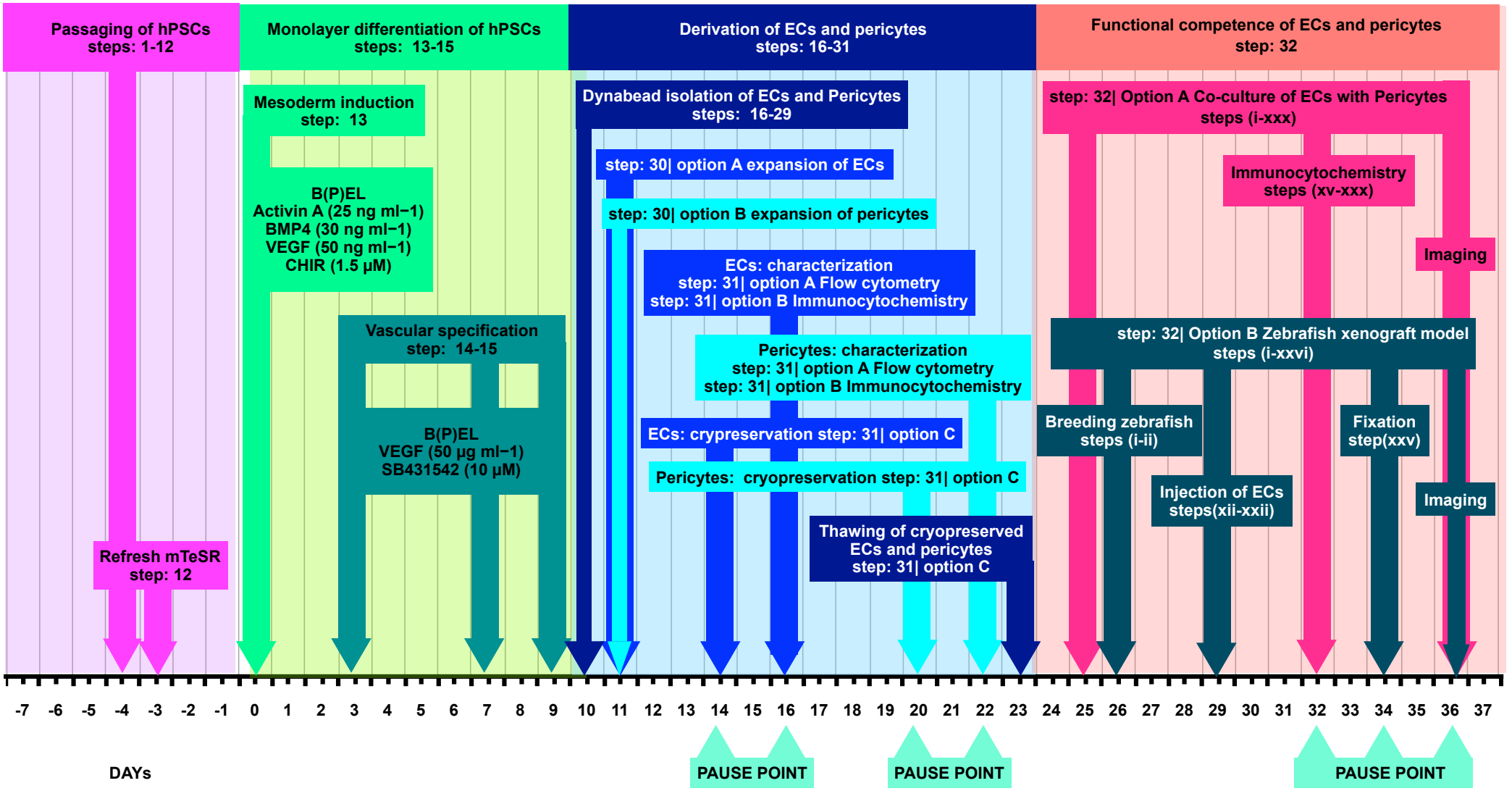
Figure 5 Co-culture of hPSC-derived ECs with pericytes.
(a) Representative image of the co-culture system at day 7, ECs are visualized with CD31 antibody (in white), (b) detection of endothelial sprouts with the AngiTool program; endothelial sprouts are shown in red and junction point is blue, (c) representative image of the co-culture system at day 7 co-stained with CD31 (in green), SM22 (in red) and DAPI (in blue), (d) detection of endothelial sprouts (in green) and SM22 positive cells (in red) with the custom CellProfiler pipeline. Scale bar 250 μm (a) and 100 μm (c).

Figure 6 Zebrafish xenotransplantation assay.
(a) Phase contrast image of zebrafish embryo prior to dechorionation. (b) During chorion removal zebrafish embryo is held with forceps and incision is made in the chorion with another set of sharp forceps. Once the chorion is pulled away the zebrafish embryo is able to freely swim out from the chorion. (c) Phase contrast image of the dechorionated embryo. (d) Schematic image of the site of ECs transplantation. Duct of Cuvier is shown with blue dashed lines, site of injection is

shown by red arrow and circle. ECs are injected ~50 μm above the position that the duct of Cuvier enters the heart. Blood leaving the heart is shown by the red dashed line. (e) Bench setup for injection showing stereomicroscope and micromanipulator. (a,b,c) Scale bar 500 μm and (d) scale bar 250 μm. Institutional regulatory board permission was obtained for the experiments in zebrafish.

Figure 7 Zebrafish xenograft as a model to assess mammalian EC functionality. (a) A representative image of the hPSC-derived ECs (in red) incorporated into the zebrafish vasculature (in green) at day 5 after transplantation. Scale bar 250 μm. (b) High magnification images of ECs (in red), (c) zebrafish vasculature (in green), (d) overplayed image at day 5 after transplantation, scale bar 20 μm. Institutional regulatory board permission was obtained for the experiments in zebrafish.

Figure 1


Passaging of hPSCs
steps: 1-12
Monolayer differentiation of hPSCs
steps: 13-15
Derivation of ECs and pericytes
steps: 16-31
Functional competence of ECs and pericytes
step: 32
Mesoderm induction
step: 13
B(P)EL
Activin A (25 ng ml−1)
BMP4 (30 ng ml−1)
VEGF (50 ng ml−1)
CHIR (1.5 μM)
Vascular specification
step: 14-15
B(P)EL
VEGF (50 μg ml−1)
SB431542 (10 μM)
Refresh mTeSR
step: 12
Dynabead isolation of ECs and Pericytes
steps: 16-29
step: 30| option A expansion of ECs
step: 30| option B expansion of pericytes
ECs: characterization
step: 31| option A Flow cytometry
step: 31| option B Immunocytochemistry
Pericytes: characterization
step: 31| option A Flow cytometry
step: 31| option B Immunocytochemistry
ECs: crypreservation step: 31| option C
Pericytes: cryopreservation step: 31| option C
Thawing of cryopreserved
ECs and pericytes
step: 31| option C
step: 32| Option A Co-culture of ECs with Pericytes
steps (i-xxx)
Immunocytochemistry
steps (xv-xxx)
Imaging
step: 32| Option B Zebrafish xenograft model
steps (i-xxvi)
Breeding zebrafish
steps (i-ii)
Fixation
step(xxv)
Injection of ECs
steps(xii-xxii)
Imaging
-7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
DAYs
PAUSE POINT
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PAUSE POINT

Figure 2

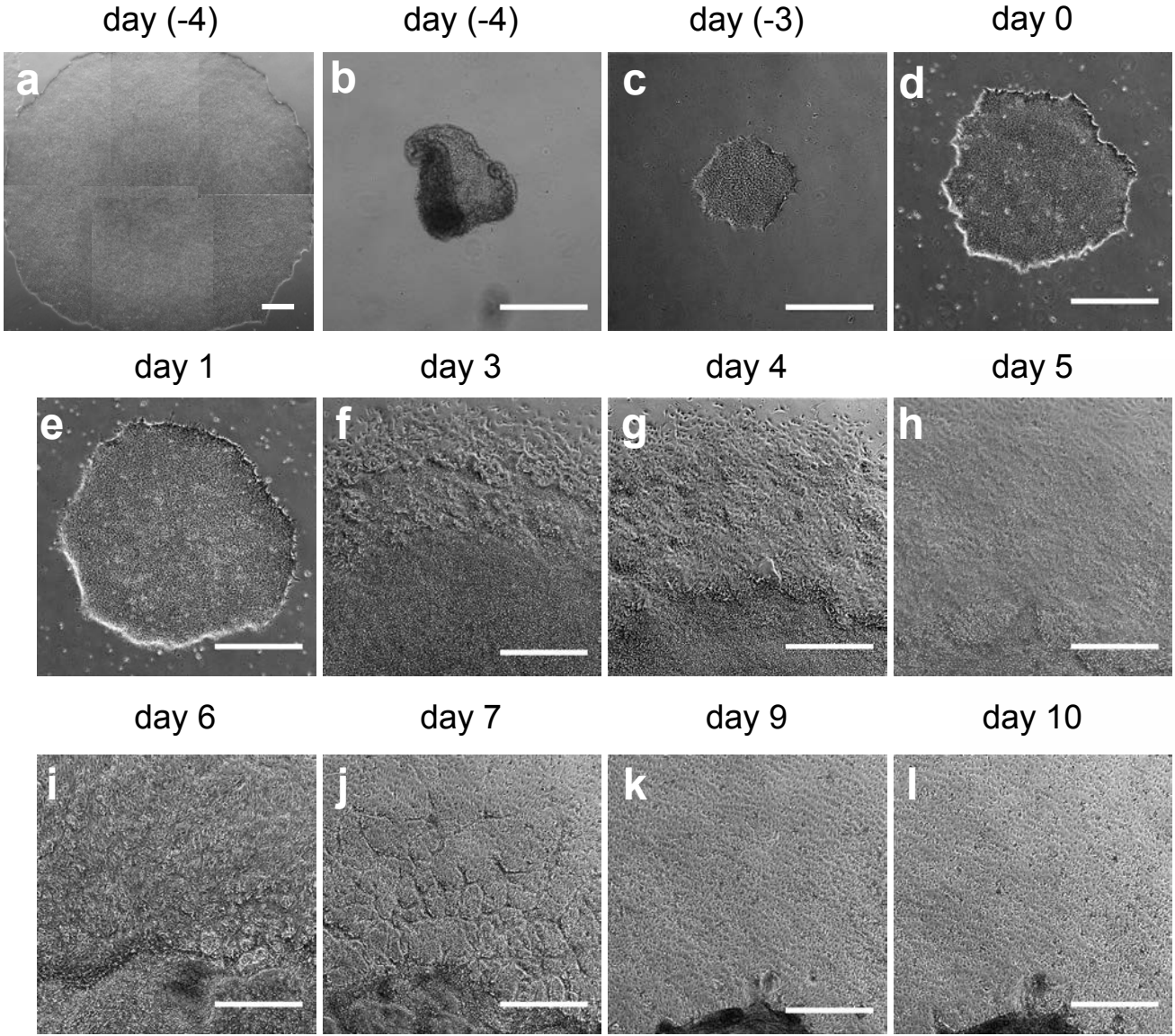
day (-4)
day (-4)
day (-3)
day 0
a
b
c
d
day 1
day 3
day 4
day 5
e
f
g
h
day 6
day 7
day 9
day 10
i
j
k
l

Figure 3

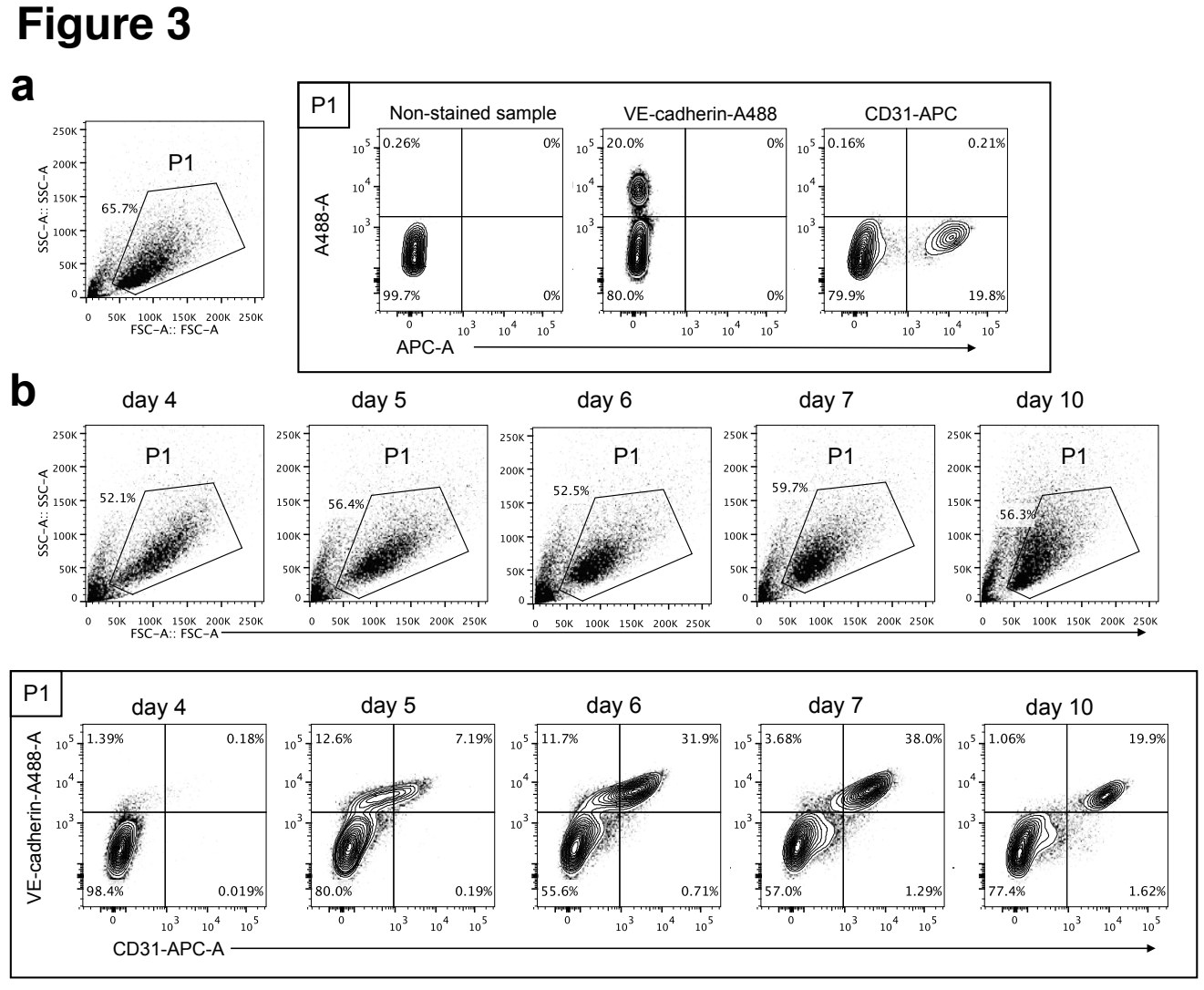
a
P1
65.7%
SSC-A:: SSC-A
FSC-A:: FSC-A
Non-stained sample
VE-cadherin-A488
CD31-APC
0.26%
0%
99.7%
20.0%
80.0%
0.16%
0.21%
79.9%
19.8%
A488-A
APC-A
b
day 4
day 5
day 6
day 7
day 10
52.1%
56.4%
52.5%
59.7%
56.3%
P1
SSC-A:: SSC-A
FSC-A:: FSC-A
P1
day 4
day 5
day 6
day 7
day 10
1.39%
0.18%
98.4%
0.019%
12.6%
7.19%
80.0%
0.19%
11.7%
31.9%
55.6%
0.71%
3.68%
38.0%
57.0%
1.29%
1.06%
19.9%
77.4%
1.62%
VE-cadherin-A488-A
CD31-APC-A

Figure 4

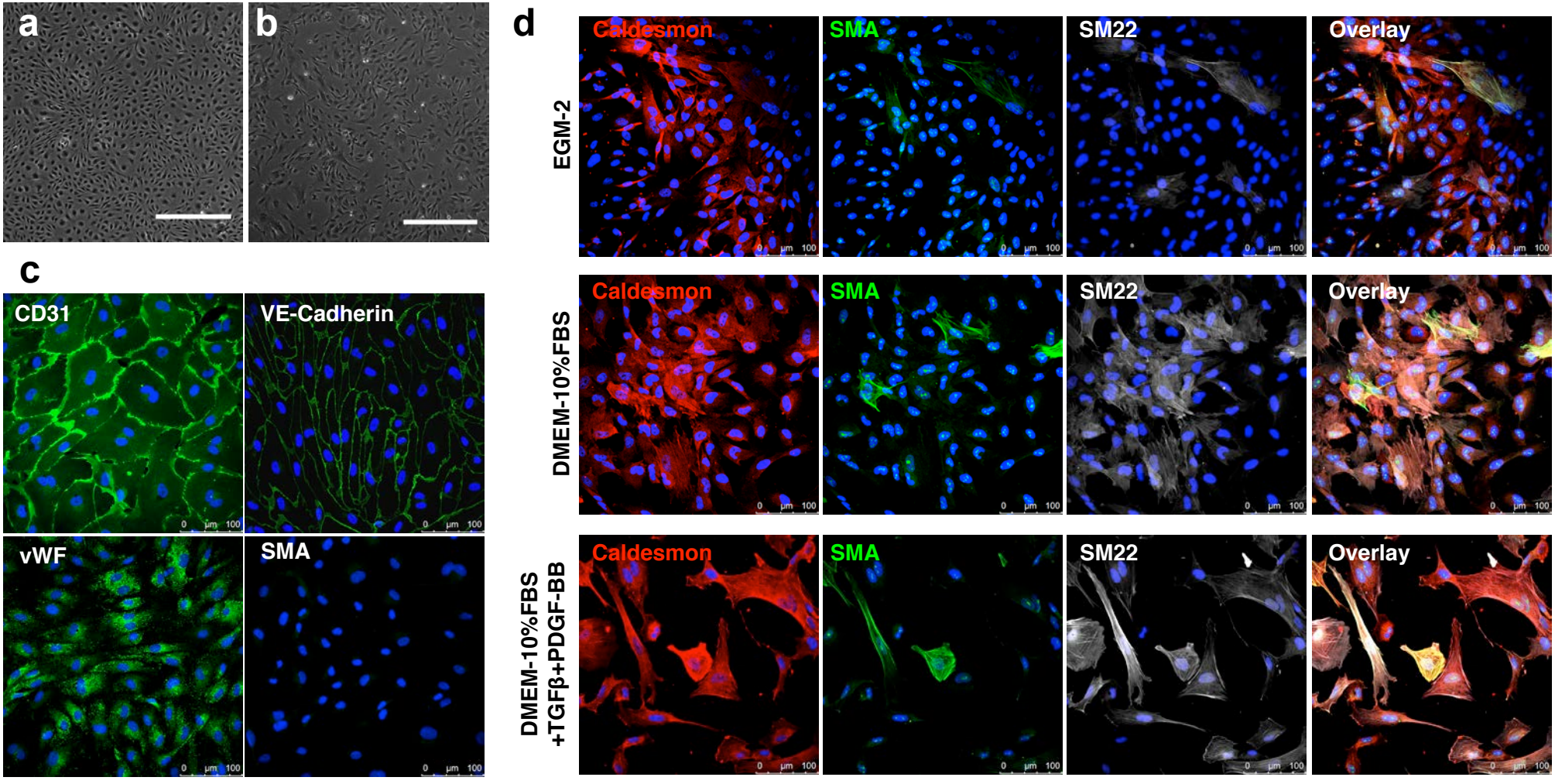
a
b
c
CD31
VE-Cadherin
vWF
SMA
d
EGM-2
DMEM-10%FBS
DMEM-10%FBS
+TGFβ+PDGF-BB
Caldesmon
SMA
SM22
Overlay

Figure 5

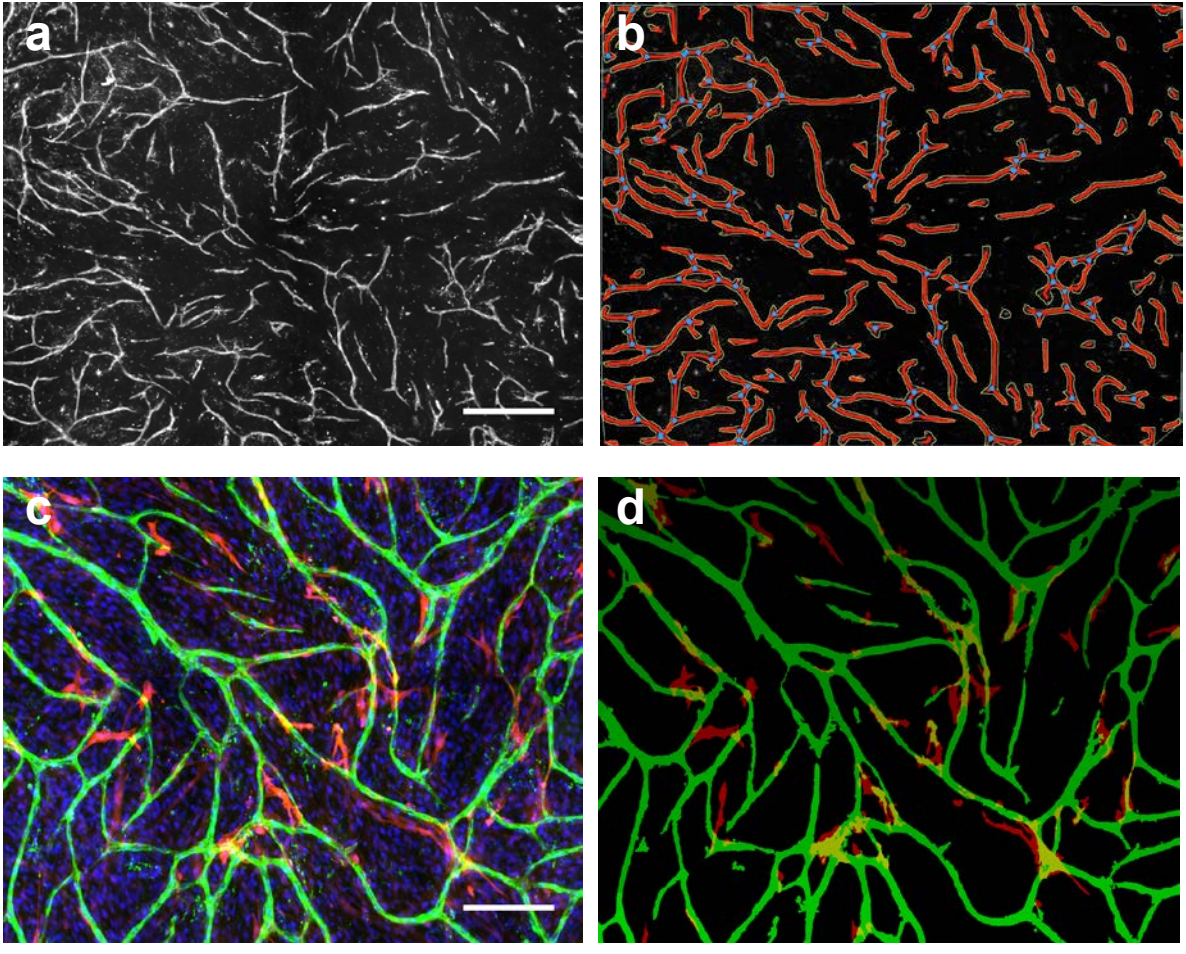

Figure 6

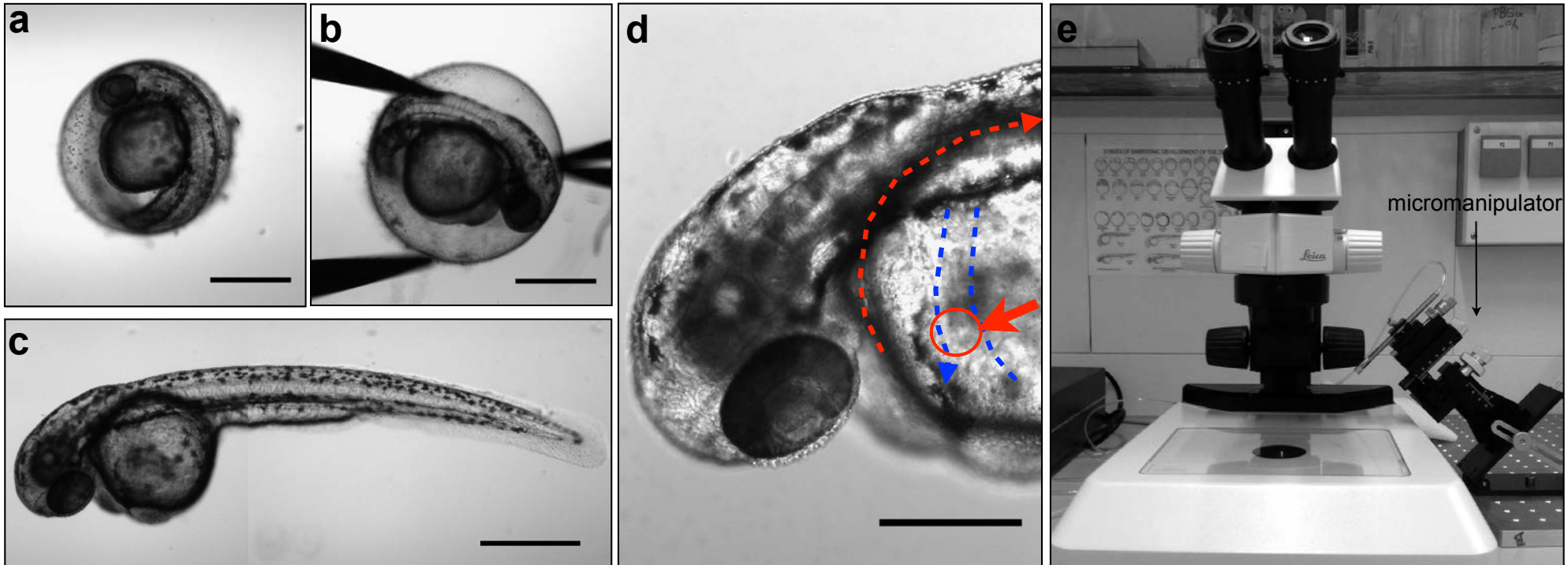

Figure 7