Isolation of endothelial cells from fresh tissues

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Here, we present a protocol for the isolation of endothelial cells (ECs) from tissues. ECs make up a minor population of cells in a tissue, but play a major role in tissue homeostasis, as well as in diverse pathologies. To understand the biology of EC, characterization of this cell population is highly desirable, but requires the availability of purified cells. For this purpose, tissues are mechanically minced and subsequently digested enzymatically with collagenase and dispase. ECs in the resulting single-cell suspension are labeled with Abs against EC surface antigens and separated from the remainder of the cells and debris by capture with magnetic beads or by high-speed cell sorting. Purified ECs are viable and suitable for characterization of diverse cellular properties. This protocol is optimized for human tissues but can also be adapted for use with other species. Depending on the tissue, the procedure can be completed in ~6 h.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is a hallmark of diverse pathologies, such as cancer, arthritis and atherosclerosis[1,2]. The progression of these diseases depends on the acquisition of new blood vessels; hence, there is substantial interest in the specific features of the endothelial cells (ECs) during these processes. Obtaining a pure population of ECs from tissues for the characterization of these cells is therefore highly desirable. However, ECs comprise only 1–2% of the total amount of cells in the tissue, and they are embedded in matrix components and tightly surrounded by various other cell types[2].

ECs express a set of surface molecules, for example CD31 and CD34, that can be used to label and distinguish the endothelium in complex cell populations[3]. In this protocol, isolation of EC is performed by making a single-cell suspension of the tissue by mechanical disruption and enzymatic digestion. As a result, Ab-labeled ECs are separated from the rest of the cells by capture with magnetic beads or using high-speed flow cytometric cell sorting (**Fig. 1**). The purified ECs can be used for various downstream applications including molecular profiling (e.g., transcriptomics[3], proteomics, micro RNA analysis, genomic analysis[4]) as well as cell culture (e.g., generation of cell lines through immortalization[5], drug testing *in vitro*[6], tissue engineering).

EC isolation methods

In recent years, purification of ECs for culture and molecular profiling has gained more and more interest, and different techniques have been employed. In early reports, tissues were minced and proteolytically digested, after which cells were seeded in culture dishes. ECs were subsequently purified by manual weeding, differential enzymatic detachment, application of selective growth media and/or the use of filters with defined pore sizes[7,8]. Alternatively, gradient density centrifugation was applied to separate ECs from other cell types (reviewed by Manconi *et al.*[8]). However, these techniques are all prone to contamination with unwanted cell types. This is detrimental to the purity of the EC population, particularly in culture when contaminating cell types may grow more rapidly and overgrow the EC. In addition, these techniques require culturing and subculturing of cells. When cells are no longer in their original environment, molecular changes will occur, which might affect the tissue specificity and integrity of the EC[9].

To overcome these limitations, a method for the direct isolation of ECs on phenotypic characteristics would be preferred[9]. After making a single-cell suspension, ECs can be separated from the bulk of the tissue by staining for EC-surface markers[10] using fluorescence-activated cell sorting (FACS)[11] or magnetic separation[12].

Although cell sorting by flow cytometry is a routinely used technique for the isolation of various cell types, such as stem cells (reviewed in ref. 13), fetal cells in the maternal circulation (reviewed in ref. 14) and gender preselection of spermatozoa (reviewed in ref. 15), the isolation of cells using high-speed cell sorting for molecular profiling is not widely used. Obviously, cell sorters are highly expensive equipment, but they offer the possibility of the simultaneous isolation of different cell populations from one sample, and the inclusion of multiple parameters per cell population[16]. In addition, the isolated cell population can directly be reanalyzed for quality control.

More widespread is the use of isolation based on Ab-coated magnetic beads. For ECs, different Abs have been used to isolate highly pure fractions of tumor ECs with beads, including anti-CD31 (refs. 3,17), *Ulex europaeus* lectin[18–20], anti-CD146[21,22], anti-VE-cadherin and anti-CD105 (refs. 23,24). The choice of the Ab, or the combination of Abs, largely depends on the target tissue. Therefore, the specificity of the Abs under consideration needs to be assessed before selection. We tested the specificity of Abs against CD31, CD34, CD105 and CD146 on tissue sections of colon tumor, normal colon and placenta and our tissues of interest[3], and demonstrated that CD31 and CD34 are the most specific Abs for EC detection (**Fig. 2**). In contrast, CD105 was expressed in placental syncytiotrophoblasts. In tumors and normal tissues, the Ab also stained stromal components in addition to the endothelium. It is surprising that although anti-CD146 Abs have been used by different groups to isolate EC, we did not observe a specific staining of endothelium in our tissue sections using an anti-CD146 Ab (**Fig. 2**).

Characterization of isolated ECs

After isolation of the desired cell population, it is very important to establish the purity of the selected population. Several endothelial markers can be examined to confirm endothelial origin. Most widely used are the expression of factor VIII-related antigen (von Willebrand factor; vWF), CD31 and CD34 (refs. 3,10,16,21,25). Using

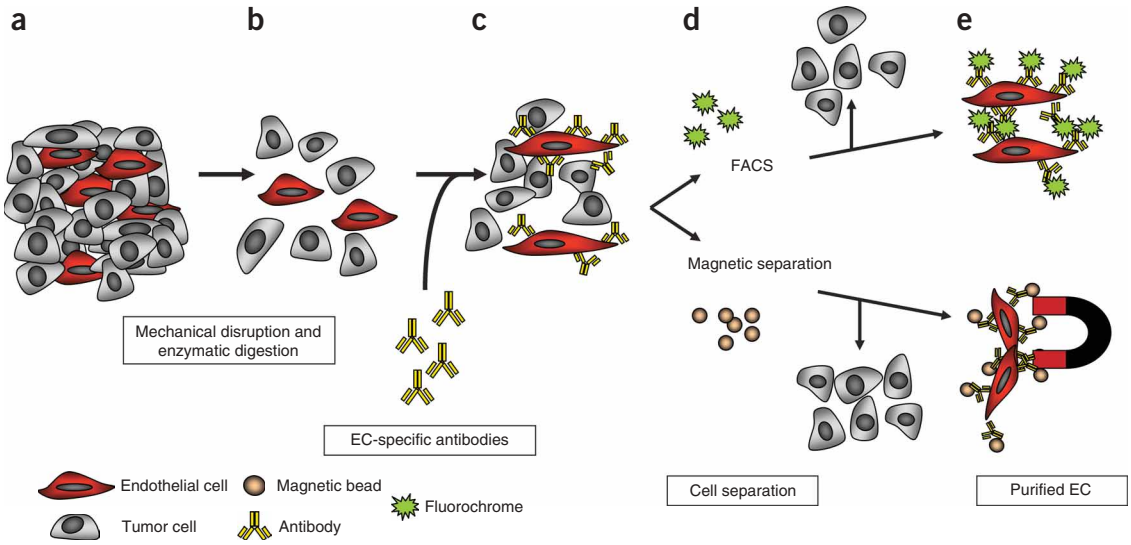

Figure 1 | Isolation of endothelial cells (ECs) from tissues by immunomagnetic separation and fluorescence-activated cell sorting (FACS). (**a**) Tissues consist of diverse cell types and matrix components, and in general the endothelial content constitutes 1–2% of the total tissue mass. (**b**) Tissues are mechanically disrupted and enzymatically digested to create a single-cell suspension. (**c**) Endothelial-specific Abs are added to the single-cell suspension to label the EC. (**d**) ECs are separated from the other components by either FACS or using magnetic beads, resulting in (**e**) highly pure population of EC.

either magnetic separation or FACS isolation, the purity of the EC fraction was >95% as established by immunostaining of isolated cells (**Fig. 3**). Reanalysis of the EC population directly after high-speed cell sorting by flow cytometry indicated ~90% purity (**Fig. 4**); however, this is an underestimation, caused by damage of the cells during the procedure and quenching of the fluorescent label by the high-power laser of the instrument. In addition—though more important when cells are propagated *in vitro*—functional properties of the EC can be addressed. These include uptake of DiI-Ac-LDL, cobblestone appearance and tube formation on 3D matrices, though these characteristics may be subjected to variation due to EC heterogeneity associated with tissue specificity[8,9].

Use of tissue-derived Ecs

Molecular profiling technologies, such as gene expression profiling and proteomics can identify biomarkers specific for a given (stage of) disease. It is well appreciated that during tumor angiogenesis, ECs undergo cellular and molecular changes that accompany the phenotypic appearance of angiogenic vessels. These molecular events induced by the malignant cells in the environment may provide sensitive markers of tumor initiation and metastasis, as well as targets for therapeutic intervention and imaging[3,22,26].

We and others have previously demonstrated that ECs stimulated with growth factors, or growing in artificial 3D matrices *in vitro* to mimic angiogenesis *in vivo*, display global gene expression profiles that are different from that of EC in tissues[3,22,26]. Hence, molecular characterization of tissue-derived ECs is of major importance to identify novel therapeutic targets and mechanisms that govern angiogenesis *in vivo*, and will provide more physiologically representative results. In addition, EC isolated from clinical samples may be used for personalized therapy decision-making and prescreening of drug efficacy. In the past, we have demonstrated the power of identification and characterization of tissue (tumor) EC by flow cytometry[3,27–29], as well as the global mRNA profiling of EC from human tumor tissues[3]. Furthermore, we have used EC isolated from murine tissues to profile-adhesion molecule expression (**Fig. 5**)[27].

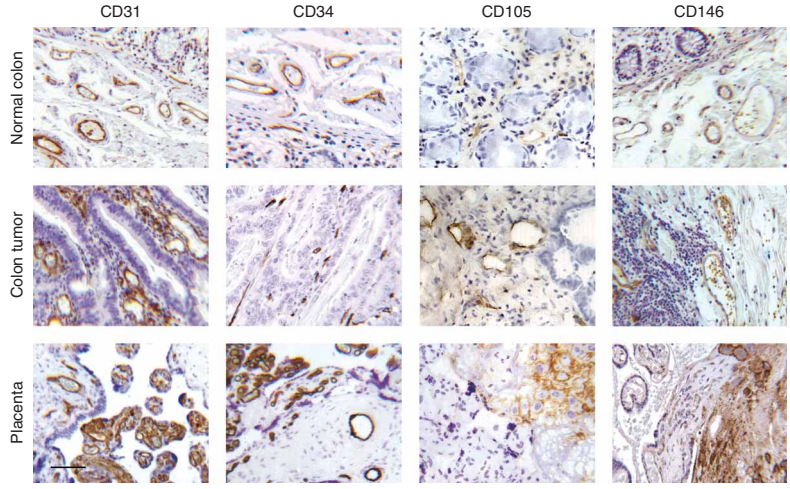

Figure 2 | Selection of Abs for endothelial cell (EC) isolation. Paraffin-embedded (CD31, CD34, CD146) or frozen (CD105) tissue sections of normal colon, colon carcinoma and placenta were stained with Abs against the indicated cell-surface markers. Anti-CD31 and anti-CD34 Abs show a specific staining of the EC throughout the tissue, confirming their suitability for EC selection. In contrast, anti-CD146 Abs show only minimal staining of blood vessels, but moreover also stain nonrelevant stromal compartments. In colon tissue, the anti-CD105 Ab stains EC and also some stromal tissue; however, in placenta tissue, predominantly non-ECs are stained. Thus, both anti-CD105 and anti-CD146 Abs were rejected for isolation purposes in these tissue types. Scale bar in the lower left panel represents 100 μm.

This protocol is optimized for use with human tissues. We have extensively used it for the isolation of EC from tumors of the kidney and colon and its corresponding normal tissue, as well as with placenta, but in principle, this procedure can be adjusted to be used with virtually every human tissue. The yield of EC will depend on the vascular density of the tissue, the expression of CD31 as well as the tissue composition which will affect the efficiency of digestion.

In addition, we have used a modified version of this protocol to isolate EC from murine tissues. As the size of these tissues are rather small, the yield of EC may not be sufficient for downstream applications directly. Hence, single-cell suspensions are expanded *in vitro* for 3–5 d and subsequently sorted for EC[27] (Castermans *et al*, manuscript submitted). However, this culture step may induce unwanted phenotypic changes in the EC.

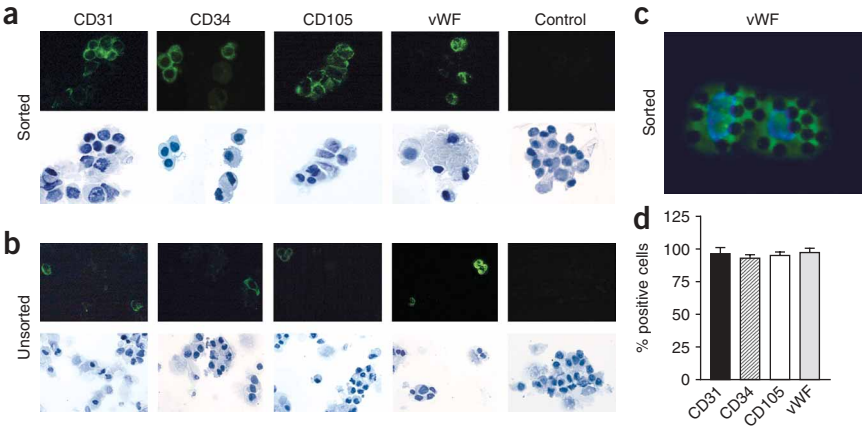

Figure 3 | Immunofluorescent characterization of isolated endothelial cells (ECs). (**a**) Cells were spun onto microscope slides and subjected to immunofluorescence staining against the indicated antigens. Purified ECs show a near complete positivity for all the antigens tested, whereas only a fraction of the cells in (**b**) the unsorted cell population is stained for the indicated antigens. Top panel: fluorescein isothiocyanate (FITC)-labeled Abs; bottom panel: hematoxylin staining. (**c**) EC captured on beads and stained for von Willebrand factor (vWF) (green) counterstained with 4,6-diamidino-2-phenylindole (DAPI) (blue). Note that multiple beads are bound to the cells. (**d**) Average percentage of positive cells for the indicated markers after EC selection.

MATERIALS

REAGENTS

- DNaseI (Sigma, cat. no. D-4527) (see REAGENT SETUP)
- Collagenase type II (Invitrogen, cat. no. 17101-015) (see REAGENT SETUP)
- Dispase (Invitrogen, cat. no. 17105-041) (see REAGENT SETUP)
- Ficoll-Paque PLUS (Amersham, cat. no. 17-1440-02) (see REAGENT SETUP)
- Mouse anti-human CD31-PE Ab (10 μg ml^{-1}; Serotec, cat. no. MCA1738PE)
- Mouse anti-human CD31 Ab (200 μg ml^{-1}; Dako, cat. no. M0823)
- Mouse anti-human CD34 Ab (200 μg ml^{-1}; Novocastra, cat. no. NCL-L-END)
- Mouse anti-human CD105 Ab (200 μg ml^{-1}; Monosan, cat. no. Mon6016)
- Mouse anti-human CD146 Ab (100 μg ml^{-1}; Abcam, cat. no. Ab22769)
- Rabbit anti-human vWF Ab (1 mg ml^{-1}; Dako, cat. no. A0082)
- Mouse anti-human CD45 Ab (500 μg ml^{-1}; Pharmingen, cat. no. 555480)
- Rat anti-mouse CD31-PE (200 μg ml^{-1}; Pharmingen, cat. no. 553373)
- Goat-anti-mouse Dynabeads (Invitrogen, cat. no. 110-33)
- RPMI-1640 (Invitrogen,cat. no. 52400-025)
- L-GIn (200 mM; Invitrogen, cat. no. 25030-024)
- Penicillin–streptomycin (10,000 U ml^{-1}; 10,000 mg ml^{-1}; Invitrogen, cat. no. 15140-122)
- Sodium pyruvate (100 mM; Invitrogen, cat. no. 11360-039)
- Nonessential amino acids (10 mM Invitrogen, cat. no. 11140-035)
- HEPES
- Buffer solution (1 M; Invitrogen, cat. no. 15630-056)
- Heparin (Leo Pharma, cat. no. 013192-02)
- Minimal essential medium (MEM) vitamins (100×; Invitrogen, cat. no. 11120-037)
- $C_6H_5Na_3O_7 \cdot 2H_2O$ (Merck, cat. no. 06448)
- $NaH_2PO_4 \cdot 2H_2O$ (Merck, cat. no. 06580)
- KH_2PO_4 (Merck, cat. no. 04873)
- FCS; heat inactivated (Hyclone, cat. no. CH30160.03)
- BSA (Sigma, cat. no. A7030)
- Gelatin type B (Sigma, cat. no. G9391) (see REAGENT SETUP)
- Human serum (HS); heat inactivated (In house preparation) (see REAGENT SETUP)
- Tissues (see REAGENT SETUP)
- Sodium citrate (see REAGENT SETUP)
- EC medium (see REAGENT SETUP)
- FACS medium (see REAGENT SETUP)

EQUIPMENT

- Centrifuge with temperature regulator suitable for 50-ml tubes
- FACS (e.g., BD FACSAria Cell-Sorting system, BD Biosciences)
- Dynal magnetic particle concentrator (MPC) for 1.5-ml tubes
- Surgical blades
- Disposable Petri dishes
- 50-ml Tubes
- 1-ml Syringes
- 50-ml Syringes
- Cell strainers (100 μm, BD Biosciences, cat. no. 352360 and 70 μm BD Biosciences, cat. no. 352350)
- FACS tubes with caps

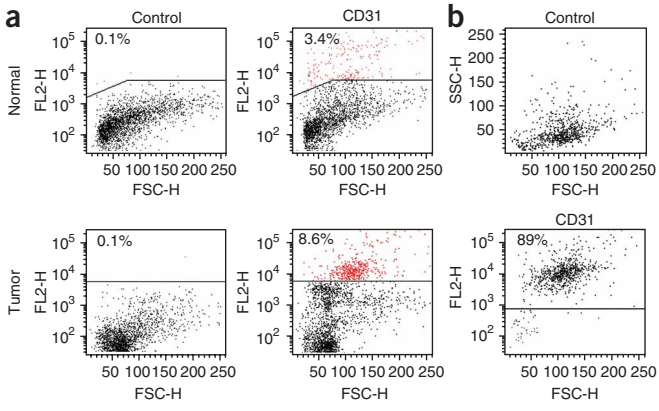

Figure 4 | Fluorescence-activated cell sorting (FACS) plots of endothelial cell (EC) isolation. (**a**) Dot plots of single-cell suspensions. Negative control samples (left panels) are used to determine the gating parameters for sorting. Gating is set at the level of fluorescence above which almost no cells are positive. Staining of the cell suspension with anti-CD31-PE Ab (right panels) shows that the ECs make up a small percentage of the total cell mass. It is interesting to note that, in general, tumor tissue contains a higher percentage of ECs, explained by the high degree of vascularization induced by the tumor cells' need. (**b**) Reanalysis of the sorted ECs shows ~90% purity. Note that the overall fluorescence has decreased as a result of the sorting procedure. FSC, forward scatter; SSC, sideward scatter.

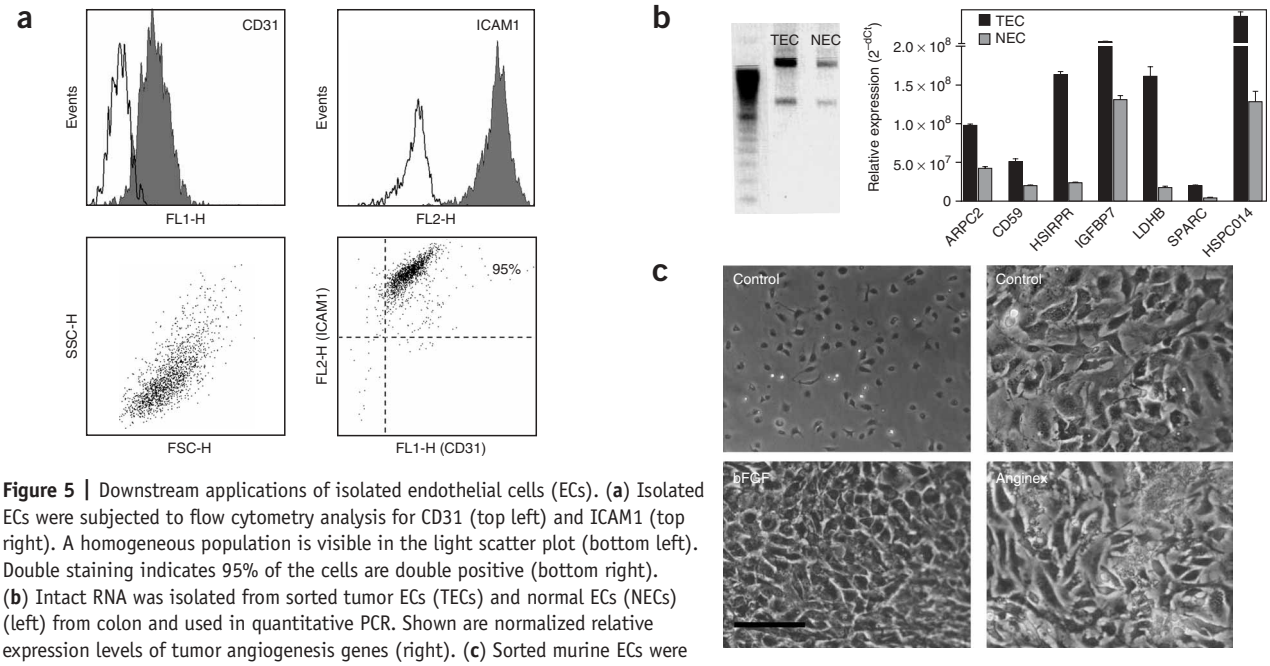

Figure 5 | Downstream applications of isolated endothelial cells (ECs). (**a**) Isolated ECs were subjected to flow cytometry analysis for CD31 (top left) and ICAM1 (top right). A homogeneous population is visible in the light scatter plot (bottom left). Double staining indicates 95% of the cells are double positive (bottom right). (**b**) Intact RNA was isolated from sorted tumor ECs (TECs) and normal ECs (NECs) (left) from colon and used in quantitative PCR. Shown are normalized relative expression levels of tumor angiogenesis genes (right). (**c**) Sorted murine ECs were cultured to test the influence of proangiogenic and antiangiogenic compounds. Shown are ECs 1 d after fluorescence-activated cell sorting (FACS) (top left) and after 4 d in culture (top right) in the presence of the proangiogenic factor basic fibroblast growth factor (bFGF) (bottom left) and the antiangiogenic compound anginex (bottom right). Scale bar in the lower left panel represents 100 μm.

• Filtration membranes for solutions: depending on the volume ZapCap CR filter (0.2 μm; Whatman/Sigma Aldrich, cat. no. Z222593) or Puradisc FP30 syringe filters (0.2 μm; Whatman/Sigma Aldrich, cat. no. F8552)

REAGENT SETUP

Tissues Collect tissue samples (preferably >1 g) as quickly as possible after excision. Samples can be collected in tubes containing EC isolation medium and can be stored at 4 °C for several hours before the start of the isolation procedure. This protocol is optimized for the use with human tissue samples, but can be applied to tissues originating from other species as well. In this protocol, we also give directions for the sorting of EC from mouse tissues. However, the EC yield of small size murine tissues may not be sufficient for downstream applications and expansion *in vitro* before and/or after cell sorting should be considered.

The EC isolation medium used throughout the procedure is regular EC medium supplemented with additional growth factors and nutrients to reduce the impact of the isolation procedure on the EC phenotype.

Abs The (combination) of Abs used for the selection of EC may vary with the tissue. It is advised to test the Abs under consideration for EC specificity in the tissue of interest, for example by immunohistochemistry on tissue sections. For the isolation of EC from normal colon, colon tumor and placenta tissues, we tested different Abs (anti-CD31 [Clone JC70A, dilution 1:50]; anti-CD34 [Clone QBend10, dilution 1:50], anti-CD105 [Clone PN-E2, dilution 1:50] and anti-CD146 [Clone OJ79c, dilution 1:100]) for their capacity to stain EC (**Fig. 2**). Furthermore, optimal dilution as well as suitability of the Ab for flow cytometry of live cells should be determined. As intact, viable cells are to be selected, Abs directed against cell-surface antigens are needed. We have used phycoerythrin (PE)-labeled mouse anti-human CD31 (1 μg ml^{-1}) for the sorting of human EC, and rat anti-mouse CD31 (2 μg ml^{-1}) for sorting of murine EC.

Dialysis of Abs Abs solutions generally contain toxic sodium azide that needs to be removed before incubation with live cells. Dialyze Abs using dialysis membranes with a molecular weight cutoff of <100 kDa overnight against PBS to remove azide, and store at 4 °C.

PBS (pH 7.2–7.4) Dissolve 8.752 g NaCl, 1.416 g $Na_2HPO_4 \cdot 2H_2O$ and 0.395 g KH_2PO_4 1,000 ml H_2O and adjust pH to 7.2–7.4. Store at 4 °C.

PBS/0.1% BSA (wt/vol) Dissolve 100 mg BSA in 100 ml PBS and store at 4 °C.

2 M sodium citrate Dissolve 6 g $C_6H_5Na_3O_7 \cdot 2H_2O$ in 10 ml PBS, and sterilize by filtration with a 0.2-μm filter. Store at 4 °C.

0.2% Gelatin (wt/vol) Dissolve 2 g gelatin in 1,000 ml PBS. Sterilize by autoclaving.

HS Provides a rich source of growth factors and nutrients for human EC and is supplemented to the media to reduce the impact of the tissue modulation on the EC phenotype. Incubate whole blood for 1 h at 37 °C and store overnight at 4 °C to allow clotting, and transfer the serum into 50-ml tubes. Spin tubes for 10 min at 700*g* to remove erythrocytes and transfer the supernatant (serum) into fresh 50-ml tubes. Spin tubes again for 10 min at 700*g* to remove any residual erythrocytes and transfer the serum into fresh 50-ml tubes. Heat-inactivate the serum (see below), cool to room temperature on ice and store at −20 °C. Q3

Heat inactivation of serum Because serum is a blood product, it contains complement, which can lead to complement-mediated cell lysis. To eliminate this risk, serum is heat-inactivated by incubating for 30 min in a 56 °C water bath, with occasional swirling to ensure equal heating. Cool the tubes on ice and store at −20 °C.

EC medium Combine 10 ml FCS, 10 ml HS, 1 ml penicillin–streptomycin (10,000 U ml^{-1}; 10,000 mg ml^{-1}), 1 ml L-GIn (200 mM) and 78 ml RPMI-1640 and filter through a 0.2-μm filter. Store at 4 °C.

Conditioned EC medium Provides a rich source of secreted growth factors from other EC grown *in vitro*. Collect medium from routinely cultured human EC (e.g., human microvascular EC, human umbilical vein EC) that are subcultured 1:3 and grown to confluence in 3 d. Filter sterilize the medium through a 0.2-μm filter and store in aliquots at −20 °C. Thawed aliquots can be kept at 4 °C for ~1 week.

EC isolation medium Consists of EC medium supplemented with additional nutrients and growth factors. Component A consists of 74 ml RPMI-1640 supplemented with 1 ml L-GIn (200 mM), 1 ml penicillin–streptomycin (10,000 U ml^{-1}; 10,000 mg ml^{-1}), 1 ml sodium pyruvate (100 mM), 1 μl heparin (1,000 U ml^{-1}), 1 ml nonessential amino acids (10 mM), 1 ml HEPES (1 M), 1 ml MEM vitamins (100×), 10 ml FCS and 10 ml HS. Component B consists of filter-sterile conditioned EC medium. Components A (80% vol/vol) and component B (20% vol/vol) are mixed and stored at 4 °C. Complete EC isolation medium can be stored at 4 °C for 1 week.

We use this rich medium for both isolation and *in vitro* expansion when this is necessary. We have observed that cultured primary human ECs generally grow better on medium supplemented with HS and conditioned medium from EC, though in the isolation protocol HS may be replaced with FCS. When mouse ECs are cultured, HS can be replaced with FCS, and conditioned medium of human EC may be replaced with conditioned medium of mouse EC, for example bEnd5.

Ficoll separation medium To prevent any residual blood clotting during the procedure, sodium citrate (1% vol/vol; 2 M) is added to EC medium for the Ficoll gradient centrifugation step.

FACS medium To preserve the functioning of the high-speed cell sorter, cells should be suspended in a solution that does not contain a high amount of additional proteins, yet cells need to remain viable during the procedure.

To balance between these conditions, cells are resuspended in RPMI-1640 supplemented with 0.1% BSA for sorting.

Collagenase solution Dissolve 100 mg collagenase II in 100 ml PBS to obtain a 0.1% collagenase solution. Store in 9-ml aliquots at −20 °C.

Dispase solution Dissolve dispase to 2.5 U ml^{-1} in PBS. Store in 1 ml aliquots at −20 °C.

DNase solution Dissolve 1 mg DNaseI in 1 ml PBS. Store in 75 µl aliquots at −20 °C.

PROCEDURE

Preparation of single-cell suspensions for EC isolation

1| Collect the tissue and weigh samples for reference. This protocol is optimized for the processing of tissue samples weighing 1 g. For smaller or larger tissues, adjust volumes accordingly.

▲ **CRITICAL STEP** To avoid the introduction of unwanted changes in gene/protein expression and the contamination with microorganisms, the processing of the tissue should be performed using sterile reagents and where applicable in a laminar flow cabinet. The samples are kept at room temperature unless otherwise indicated.

■ **PAUSE POINT** If necessary, tissue is stored before processing overnight in EC isolation medium at 4 °C.

2| Mince tissue using two surgical blades in a Petri dish (as finely as possible, though pieces should be at least $<1\ mm^3$) and put mashed tissue into 50-ml tubes with 9 ml collagenase and 1 ml dispase solution per gram of tissue. Incubate the tissue suspension for 30 min in a 37 °C water bath, under continuous agitation. Subsequently, add 75 µl DNaseI solution per 10 ml cell suspension and incubate for another 30 min in a 37 °C water bath with continuous agitation. Tissues can vary in their composition which affects the efficiency of digestion and the generation of a single-cell suspension necessary for Ab incubation. Also, care must be taken not to include necrotic tissue that increases the amount of debris and lowers the cell yield.

▲ **CRITICAL STEP** Improper digestion of the tissue results in the presence of cell clumps that cannot pass the cell strainer in Step 3, resulting in a decreased cell yield. To improve digestion of the tissue, the suspension can be pipetted up and down every 5 min.

? TROUBLESHOOTING

3| Put the tube on ice and add 5 ml cold EC isolation medium for every 10 ml of collagenase/dispase solution to the tissue suspension. Sieve the sample through a 100-µm cell strainer fitted on a 50-ml tube on ice to remove undigested cell clumps and separate the single cells. Use the plunger of a 1-ml syringe to press the suspension through the filter. Rinse filter with 5 ml cold EC isolation medium. Collect cells by centrifugation at 400*g* for 5 min at room temperature.

? TROUBLESHOOTING

4| To remove red blood cells, granulocytes, nonvital cells and cell debris, resuspend cells in 10 ml Ficoll separation medium (per gram of starting material) and carefully layer the suspension on 7.5 ml Ficoll-Paque (prewarmed to room temperature) in a 50-ml centrifugation tube. Spin at 400*g* for 20 min at 18 °C without brakes.

▲ **CRITICAL STEP** Layer the cell suspension carefully on the Ficoll as disturbance of the Ficoll-Paque will compromise gradient density centrifugation. As the Ficoll may induce changes in the EC, put on ice immediately after centrifugation, work quick and retrieve all the interphase cells with a minimum amount of Ficoll. Be sure to centrifuge without brakes as deceleration will disrupt the density gradient.

5| Transfer the interphase containing viable cells into a fresh tube and add 2 volumes of EC isolation medium. For easy collection of the cells in the interphase, first carefully remove most of the medium in the upper phase. Spin at 400*g* for 5 min to remove residual Ficoll-Paque and resuspend in the appropriate medium depending on the downstream application (see below).

EC isolation

6| Proceed with cell staining for sorting by either FACS (option A) or magnetic cell selection (option B):

(A) FACS

(i) Resuspend the cells in FACS medium and count the number of cells. Transfer cells into FACS tubes, take an aliquot of 20,000 cells as a control sample for the staining, and incubate the remainder of the cell suspension with dialyzed mouse anti-human CD31-PE (dilution 1:10; final concentration 1 µg ml^{-1}) at up to 10^7 cells ml^{-1}. Incubate cells on ice in the dark for 45 min.

(ii) Wash cell suspension once in cold FACS medium (spin at 400*g* for 5 min).

(iii) Take up cells at 10^7 cells ml^{-1} in FACS medium and filter through a 70-µm mesh filter.

(iv) Prepare the FACS machine by adjusting settings and gates for isolation of pure EC. Define the negative population by the control sample (**Fig. 4**).

(v) Collect the cells in 4-ml FACS tubes containing 3 ml EC isolation medium. Cool both the sample chamber and the collection tubes to 4 °C throughout the sorting procedure.

▲ **CRITICAL STEP** Improper gating of the positively stained population is detrimental to the purity of the sorted fraction. Cell survival and purity of sorting are strongly influenced by the cell sorting rate, the sorting time and the pressure of the sheath fluid. As ECs in suspension are rather fragile, the default BD FACS Aria presets for sorting conditions need to be

adjusted. The customized setup is characterized by a sheath fluid pressure of 29.9 psi, a sorting frequency of 44 kHz and a plate voltage of 3,500 V. Laser delay, area scaling factor and drop delay are set accordingly. To facilitate high-speed sorting and to prevent clogging of the nozzle, the cell suspension should be absolutely clear of cell clumps, and should be diluted such that at a flow rate of 2.0 (which corresponds to ~20 µl min^{-1}) an event rate of 5,000 s^{-1} is not exceeded.
? TROUBLESHOOTING

(B) Magnetic cell selection

(i) Resuspend the cells in EC isolation medium and count the number of cells. Transfer cells into 10-ml tubes at a concentration of up to 10^7 cells ml^{-1}. Add dialyzed anti-CD31 Ab (Dako) to a concentration of 10 µg ml^{-1} to the single-cell suspension and incubate on ice for 45 min.

(ii) Wash cell suspension in cold EC isolation medium and spin at 400*g* for 5 min. Resuspend cells in 1 ml EC isolation medium.

(iii) Prewash secondary Ab-coated Dynabeads with EC isolation medium. Add Dynabeads at an approximate ratio of four beads per EC (assume 5% of the cells are ECs). Incubate on a rotator for 45 min at 4 °C.

(iv) Transfer the cell suspension into 1.5-ml tubes and place in the MPC.

(v) Allow magnetic beads to concentrate at the side of the magnet. Remove unbound cells carefully.

(vi) Remove tube from rack and wash cells with cold EC isolation medium by inverting the tube gently several times. Place tube again in MPC.

Q4 (vii) Repeat wash Step 6B(v and vi) four times. Collect bead-bound cells.
▲ **CRITICAL STEP** Be sure not to remove the vial from the magnet during collection of the unbound cells as this will result in reduced yields. Cells are collected when still bound to the beads, but can directly be used for downstream applications that require cell lysis.

Quality control

7| Collect an aliquot (20,000 cells) of the single-cell suspension before EC isolation and after selection of cells. Cells sorted by FACS can be reanalyzed by flow cytometry directly.
? TROUBLESHOOTING

8| Cells isolated by either method can be subject to immunocytochemistry to determine the purity of the isolated fraction. Prepare cytospins of the cells and stain preparations with anti-endothelial Abs, for example, directed against vWF (dilution 1:200), CD105, CD31 and/or CD34. Count the percentage of positive cells (**Fig. 3**).

● **TIMING**

To preserve the features of EC physiology and gene expression, the tissue specimen should preferably be processed immediately after resection. It is advisable to have ready-to-use aliquots of the work solutions in storage. If necessary, tissue samples can be stored overnight in EC isolation medium at 4 °C.

Preparation of the single-cell suspension for incubation with Abs will take ~2.5 h. Ab incubation takes 1 h. FACS analysis and the preparations thereof largely depend on the specifications of the equipment as well as on the number of ECs in the sample. Assuming a sort rate of 5,000 events s^{-1} and the presence of 3% labeled EC in the single-cell suspension, isolation of 10^6 EC will take ~2 hours. Concentration of magnetic bead-bound cells to the side of the MPC takes a few seconds, consecutive washes take up to 30 min depending on the starting sample size.

? TROUBLESHOOTING

Step 2

In our hands and with the tissues we use, the two times 30-min digestion is sufficient to digest the tissue in such a way that all tissue can be sieved through the cell strainer, except some fragments of the connective tissue. However, for use with other tissues, the number of digestion times might need to be adjusted to prevent overdigestion or improper digestion.

Step 3

Filters may need to be changed repeatedly as a result of clogging.

Step 6A

Gates are defined based on the fluorescence of samples that were stained with the relevant Abs and control cells (**Fig. 4**). Minor adjustments to the settings of the sorter may be necessary as they may be, to a minor extent, dependent on the apparatus.

For the sorting of mouse EC, PE-labeled rat anti-mouse CD31 (dilution 1:100; end concentration 2 µg ml^{-1}) is used. If the expected yield of EC is not sufficient for direct downstream applications, adjust the sorting parameters for maximum viability of the sorted cells, and grow the EC in EC isolation medium in 0.2% gelatin-coated tissue culture plates. Plate cells at a density of 30,000 cells cm^{-2}.

Step 7

Reanalysis of sorted cells by flow cytometry will provide direct information on the purity of the isolated cells. However, this is likely to give an underestimation as the fluorochrome may have been quenched by excitation with the laser for FACS, and some cells may have been damaged and fall outside the gates.

ANTICIPATED RESULTS

From 1 g of fresh tissue, $\sim 10^6$ ECs can be sorted. However, this depends largely on the composition and architecture of the tissue. Approximately 1–2% of the cells of a tissue are ECs, although in highly vascularized tissues, such as certain tumors, this can be substantially higher (**Fig. 4**). Purity of the sorted cell population is typically $>95\%$ (**Fig. 3**), although this depends highly on stringency of the sorting parameters applied to the cell population of interest. Alternatively, cells can be isolated using paramagnetic beads with comparable results.

We have used CD31 to identify EC for sorting, though certain subsets of leukocytes are also known to express CD31. However, these cells are smaller than ECs and the expression of CD31 is much higher on EC than on other hematopoietic cells[30]. As such, stringent gating on light scatter characteristics and fluorescence parameters can exclude these unwanted cells from the sorted population. Indeed, we did not observe many CD45 positive cells in cytospins of sorted cells. Furthermore, we have previously shown that leukocyte infiltration is downregulated both in the tumor and in the control organs in tumor-bearing animals[27,31], further reducing the possibility of isolating unwanted cells from tissues from cancer patients.

We observed high viability of the sorted cells ($>95\%$) as determined by dye exclusion (trypan blue). The sorted cells are suitable for various downstream applications such as gene and protein expression profiling, cell culture and drug testing (**Fig. 5**). As the sorted cells may induce phenotypic changes during cell culture, it is highly recommended to use the sorted cells immediately when molecular profiling is envisaged. However, if the cell number is limiting, cells can also be expanded *in vitro* after sorting. For culture to be successful, cell sorting should be performed under sterile conditions, and sorting parameters (mainly sheath fluid pressure) and speed should be optimized for viability of the cells. As described in REAGENT SETUP, media containing additional nutrients and conditioned medium from cultured ECs are used to grow the sorted EC.

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