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Supplemental Information

Human Teratoma-Derived Hematopoiesis Is a Highly Polyclonal Process Supported by Human Umbilical Vein Endothelial Cells

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Figure S1. Flow cytometric analysis of teratoma samples and generation of hematopoietic supporter cell types. Related to Figures 1 and 2 .

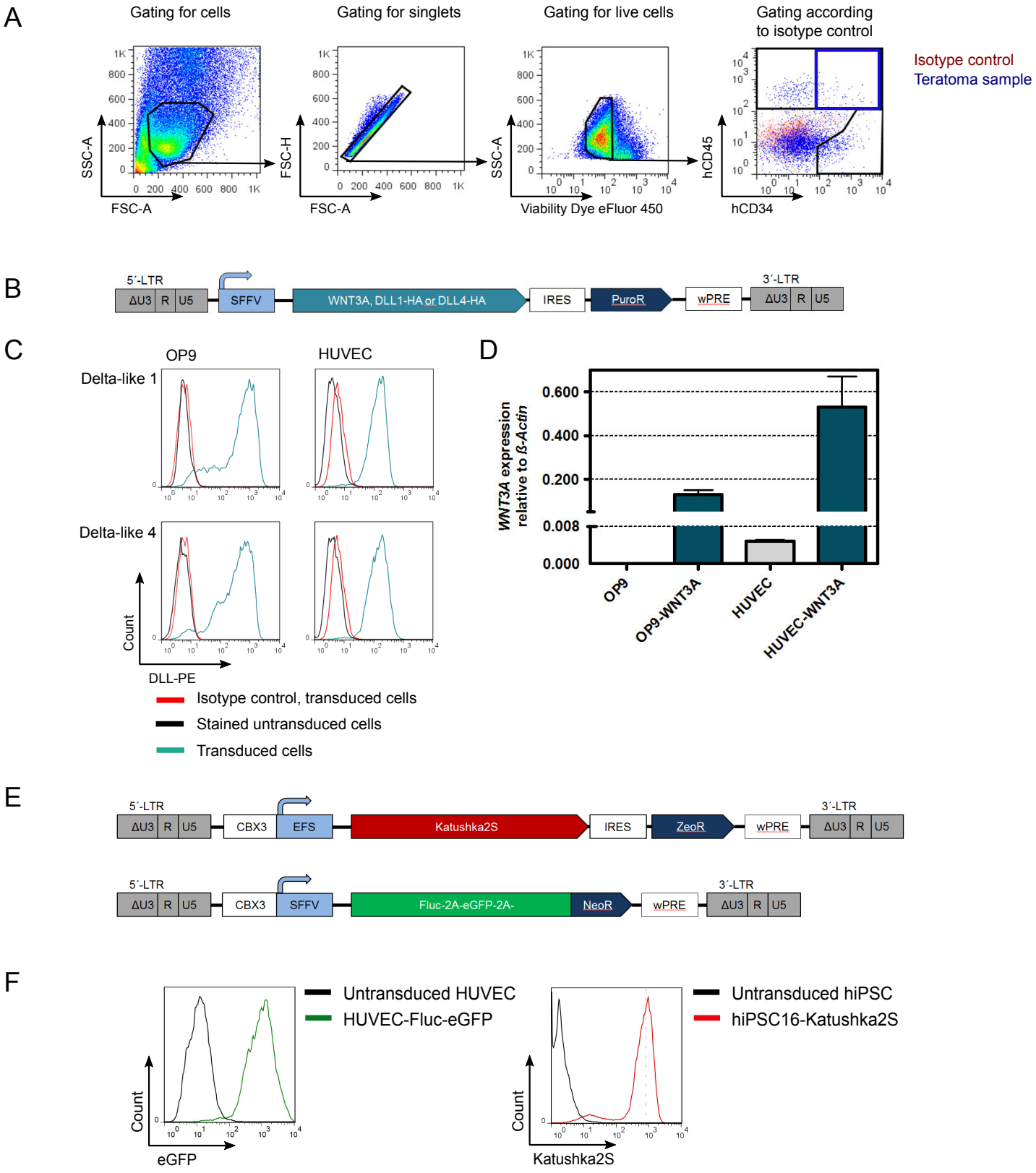

Figure S1. Flow cytometry (FC) analysis of teratoma samples and generation of hematopoietic supporter cell lines. Related to Figures 1 and 2. (A) Gating strategy for FC analysis of hematopoietic cells in teratoma samples. After cells were gated in SSC/FSC, doublets were excluded. Then, living cells were selected by viability staining. Gates were set according to isotype controls. (B) Lentiviral vector for the overexpression of DLL1, DLL4 and WNT3A in OP9 and HUVEC. (C) Notch ligand overexpression in transduced OP9 or HUVEC prior to co-injection for teratoma formation. Untransduced OP9 and HUVEC were stained as negative controls. (D) Quantitative PCR to determine *WNT3A* mRNA levels in HUVEC-WNT3A or OP9-WNT3A. *WNT3A* expression was related to *ß-Actin* mRNA level. Graph shows technical replicates n=3, mean and SD. (E) Lentiviral constructs for Katushka2S and firefly luciferase (Fluc) expression. (F) Expression analysis of transduced HUVEC-Fluc-eGFP and hiPSC-Kat by FC. Untransduced cells were used as controls.

Figure S2. Identification of hematopoietic, endothelial and mesenchymal cells by immunohistochemistry. Related to Figure 3.

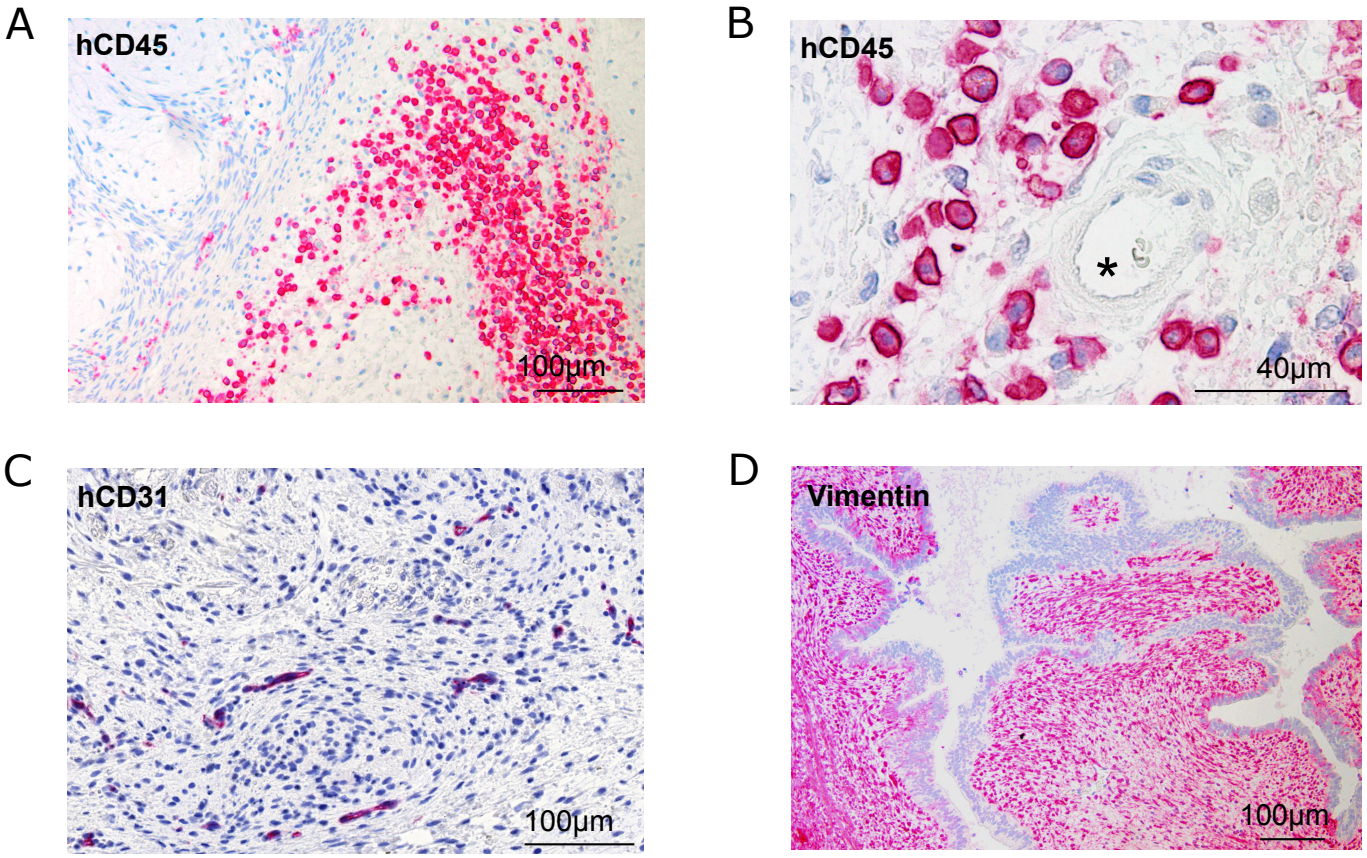

Figure S2. Identification of hematopoietic, endothelial and mesenchymal cells by chromogen immunohistochemistry (IHC) on hiPSC-derived teratomas generated in NSGS mice. Related to Figure 3. (A) Staining of human hematopoietic marker CD45. (B) Hematopoietic cells (CD45) in teratoma tissue close to a blood vessel which is labeled with *. (C) Human endothelial cells, marked by CD31. (D) Staining of mesenchymal marker Vimentin.

Supplemental experimental procedures

Cell culture

Human iPSC were co-cultured on murine embryonic fibroblasts C3H (kindly provided by T. Cantz, Hannover Medical School) according to standard protocols. Unless stated otherwise, the iPSC clone CD34iPSC16 was used (Lachmann *et al*., 2014). hiPSC cultures were maintained in Knockout DMEM (Gibco) supplemented with 20 % KO serum replacement (Gibco), 1 % NEAA (Gibco), 2 mM glutamine (Biochrom), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (PAA) and 20 ng/ml bFGF (kindly provided by the Department of Technical Chemistry, Leibniz University Hannover). OP9 and HUVEC (VeraVec, Angiocrine Bioscience) were cultivated on 0.1 % gelatin coated flasks. Detachment was done with Trypsin/EDTA (Pan Biotech). OP9 cells were cultured in αMEM Medium +GlutaMAX-I (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (PAA) and 15 % fetal bovine serum (Brazil One, Pan Biotech). HUVEC were cultured in Medium199 (Gibco), 20 U/ml Heparin (Ratiopharm), 10 mM HEPES buffer (PAA), 2 mM glutamine (Merck), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA) and 20-40 µg/ml endothelial cell growth supplement (Sigma-Aldrich).

Lentiviral constructs

Lentiviral vectors for Notch ligands and WNT3A expression were constructed by using a 3rd generation lentiviral vector (kindly provided by L. Naldini, Instituto Scientifico San Raffaele, Italy) equipped with the SFFV U3 promoter (Schambach et al., 2006) by inserting the human DLL1, DLL4 and WNT3A cDNAs amplified via PCR as AgeI and SalI fragments. A 3' HA-tag was included for both DLL fragments. Thereafter, an IRES.Puromycin resistance cassette was introduced as a SalI and XhoI fragment into the vector. Final vectors pRRL.PPT.SFFV.DLL1-HA.IRES.PuroR.pre, pRRL.PPT.SFFV.DLL4-HA.IRES.PuroR.pre and pRRL.PPT.SFFV.WNT3A.IRES.PuroR.pre were used for viral supernatant production. The design and generation of barcoded vectors has been described before (Cornils et al., 2014). The vector was modified by exchanging the spleen focus forming virus promoter with elongation factor 1α, short CBX3 element (CBX3.EFS), to prevent gene silencing (Hoffmann et al., 2017; Müller-Kuller et al., 2015). The lentiviral vector for firefly luciferase (Fluc) expression, combined with Neomycin resistant gene and eGFP, was cloned as a three fragment ligation after PCR amplification of FLuc.T2A.eGFP (kindly provided by S. Waddington) (Buckley et al., 2015) as BamHI and XbaI fragment and P2A.Neomycin as XbaI and SalI fragment into the lentiviral pRRL backbone with CBX3 element (pRRL.PPT.CBX3.SFFV.FLuc.2A.eGFP.2A.NeoR.pre). For Katushka2S expression (Luker et al., 2015), the Katushka2S nucleotide sequence was synthesized by GeneArt (Thermo Fisher Scientific) as a fragment flanked by BamHI and MluI restriction sites. The Katushka2S fragment and an IRES.Zeocin resistance cassette as MluI and SalI fragment were cloned into the lentiviral pRRL vector containing the CBX3.EFS promoter (pRRL.PPT.CBX3.EFS.Katushka.IRES.ZeoR.pre). Further cloning details are available on request.

Virus production

Virus production was performed with a lentiviral four-plasmid split packaging system and calcium precipitation as described before (Maetzig et al., 2014), using vector as well as gag/pol, envelope and VSVg helper plasmids. For the barcode constructs, virus titrations were performed as described elsewhere (Kraunus et al., 2004). Briefly, HT1080 cells were transduced and cultured until isolation of genomic DNA with QIAampDNABlood Mini Kit (Qiagen). Vector copy number (VCN) was determined by multiplex qPCR on viral woodchuck hepatitis virus post regulatory element and genomic PTBP2 (Rothe et al., 2012). Quantitative PCRs were performed on the ABI StepOne Plus (Applied Biosystems).

Transductions

All lentiviral transductions were performed in the presence of 4 µg/ml protamine sulfate (Maetzig et al., 2014). For transduction of hiPSCs with barcode constructs, cultures were treated with 10 µM Y-27632 one hour before harvest with Trypsin/EDTA (PAA). Cells were incubated together with the respective amount of virus supernatant (MOI= 1.5) for one hour at 37°C. Cells remained resuspended by tipping the tube every 15 minutes. Afterwards, cells were seeded onto wells coated with 0.25 % Geltrex (Thermo Fisher Scientific). Medium change was performed after 6-12 hours and contained 10 µM Y-27632. Transduced cells were sorted by FACS by means of fluorescent protein expression and kept as monolayers. Conditioned medium for monolayers was prepared by incubating hiPSC medium for 24 hours on C3H cultures followed by filtration through a 0.22 µm syringe filter (Millipore). For transduction of HUVEC and OP9, cells were plated with 30 % confluency on 6 well plates one day before transduction. The next day, medium was replaced by viral supernatant and incubated for 4-6 hours followed by a medium change. Transduced cells were selected by puromycin (Invivogen, OP9 7 µg/ml, HUVEC 3 µg/ ml) or neomycin (HUVEC 500 µg/ml). Katushka2S expressing hiPSC colonies were picked and checked for transgene expression by flow

cytometry. Nucleotide barcode labeled hiPSC were sorted for fluorescent reporter expression. Vector copy numbers were determined as described for virus transduction earlier.

qPCR to determine *WNT3A* expression
Cell cultures were harvested and RNA was isolated with RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. RNA (600 ng) was transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was accomplished with SYBR Green (Qiagen) and a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Primer for *WNT3A*, murine and human β-*Actin* amplification were described before (Galla et al., 2011, 2013; Mazzotta et al., 2016). 58°C was used as annealing temperature. Expression differences were calculated by ΔΔCt method (Pfaffl, 2001). Initial murine *Wnt3a* level in OP9 was not determined.

Mice
NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) and NSGS (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ) mice were kept in pathogen-free environment with access to food and water at the animal facility of Hannover Medical School. Females between 7 and 21 weeks were used for teratoma assays. Both strains were bred at Hannover Medical School according to animal protection laws.

Flow cytometry (FC)
Teratoma cells were incubated with Fixable Viability Dye eFluor™ 450 (Thermo Fisher Scientific) and human and murine Fc block TruStainFcX for 30 minutes on ice in the dark (#101320, #422302, Biolegend). Antibodies and corresponding isotype controls are listed below. Data was acquired with LSRII or FACSCalibur (both BD Bioscience). Data analyses were performed with FlowJo software (TriStar). Viability staining was not used for FC of cell cultures. All other staining and FC protocols were as described above.

Antibodies used for flow cytometry analysis:

Antibody for flow cytometry	Catalog number	Manufacturer	Isotype
anti-human CD34-APC	343510	Biolegend	Mouse IgG1, κ
anti-human CD34-PE/Cy7	343516	Biolegend	Mouse IgG1, κ
anti-human CD45-BV570	304033	Biolegend	Mouse IgG1, κ
anti-human CD45-FITC	304006	Biolegend	Mouse IgG1, κ
anti-human CD43-PE	12-0439-42	eBioscience	Mouse IgG1
anti-human CD31-PE	130-092-653	Miltenyi	Mouse IgG1, κ
anti-human DLL1-PE	346403	Biolegend	Mouse IgG1, κ
anti-human DLL4-PE	346505	Biolegend	Mouse IgG1, κ
Isotypes			
Mouse IgG1-APC	400120	Biolegend	
Mouse IgG1 -PE/Cy7	400126	Biolegend	
Mouse IgG1-BV570	400159	Biolegend	
Mouse IgG1-FITC	400110	Biolegend	
Mouse IgG1-PE	400112	Biolegend	

Longitudinal study
NSGS mice of 7-13 weeks were anesthetized with 2-3 % isofluorane (CP Pharma) and the lower back was shaved with an electric shaver. Teratoma inductions were carried out as described above. Luciferin (Intrace Medial SA) application was done by injection by tail vein injection (150 µg/ g, 100 µl/ 20 g in PBS). Images were captured approximately 10 minutes after luciferin injection using an IVIS SpectrumCT and software Living Image 4.5.5 (both PerkinElmer). Katushka2S was imaged with a 605/660 nm filter set.

Clonogenic assays
Teratoma cells were sorted with BD FACSARIA Fusion and software BD FACSDIVA (both BD Bioscience) for $CD34^+/CD45^+$ cells, following the gating strategy of FC analysis. Freshly isolated $CD34^+$ cord blood (CB) cells were used as positive control. CB was obtained after written consent. Mononuclear cells were separated with a densitiy gradient using Biocoll Separating Solution (Biochrom) and 50 ml LeucoSEP tubes (Fisher Scientific) according to the manufacturer's protocol. Isolation of $CD34^+$ cells was done with CD34 MicroBead Kit (Miltenyi, 13046702). Cells were counted and up to 2500 teratoma cells and up to 2000 CB cells were seeded in 1.5 ml Methocult Optimum 4034 (STEMCELL Technologies) with 100 U/ml penicillin and 100 µg/ml streptomycin (PAA) (Corning).

Colonies were scored after 14 days of incubation. Colonies of the same CFU type were then picked and pooled for cytospins.

Cytospin and analysis
Cells were centrifuged in 150 µl for 10 minutes at 450 rpm by a ThermoShandon Cytospin 4 cytocentrifuge (Thermo Fisher Scientific). Staining was performed according to the Pappenheim protocol. For that, slides were incubated in May-Gruenwald staining solution (Carl Roth) for 5 minutes. Afterwards, slides were washed in PBS and left in Giemsa solution (Sigma-Aldrich) for 15 minutes. Samples were mounted using Roti®Histokitt (CarlRoth). Pictures were taken with a BX51 microscope, camera XC50 and software Cell^F version 3.4 (all Olympus).

Immunohistochemistry (IHC)
Isolated tissue was fixed overnight at room temperature in 4 % neutral buffered formaldehyde (Carl Roth). Then, samples were embedded in paraffin and sliced in 3 µm sections. After deparaffinization, antigen retrieval was achieved by heat and citrate buffer. All antibodies and dilutions are listed below. Prior to staining, specimens were blocked using donkey (Jackson ImmunoResearch Inc) or goat serum (Vector Laboratories Inc). Incubations with primary antibody were conducted overnight at 4°C. In case of fluorescent staining, secondary antibodies were incubated for four to six hours at room temperature. Nuclei were stained with TO-PRO-3 Iodide (Thermo Fisher Scientific). Samples were mounted with Immunoselect Antifading mounting medium (Dianova). For 3D imaging of vasculature in teratoma, tissue was fixed in 2 % formaldehyde/ PBS overnight at 4°C, embedded in 2 % agarose blocks and sliced in 200 µm slices with a vibratom (Campden Instruments). During staining procedure, slices were kept in 300 µl PBS solution in 12 well plates. The same dilutions of antibodies were used as for IHC described above, but incubation times were increased to overnight for blocking and to 24 hours for antibodies at 4°C. Mounting was done with ProLong™ Gold Antifade Reagent (Thermo Fisher Scientific). Pictures were taken with Confocal LSM Meta 512 and ZEN 2009 and processed with software AxioVision SE64Rel.4.9 (all Zeiss) and Imaris Version 7.6.5 (Bitplane AG). For chromogen IHC, we applied a routine method using the Dako REAL™ Detection System and Alkaline Phosphatase/RED (Dako) staining kit. The slides were finally counterstained with Mayer's hematoxylin (Merck) and mounted with xylol (CG Chemikalien) or Eukitt (Sigma-Aldrich). Pictures were taken with a BX51 microscope outfitted with an XC50 camera and Cell^F version 3.4 software (all Olympus).

Antibodies and dilutions used for immunohistochemistry (IHC) on paraffin-embedded tissue:

1st antibody for IHC	Catalog number	Manufacturer	Applied dilution
anti-human CD34	AF7227	RD Systems	1:50
anti-human CD45	M0701	Dako	1:100
anti-human CD31	ab76533	Abcam	1:100
anti-human vimentin V9	M0725	Dako	1:100
anti-human CD43	NBP2-33746	Novus	1:80
Isotypes			
Sheep-IgG, polyclonal	5-001-A	RD Systems	According to 1st antibody
Rabbit-IgG, monoclonal	ab172730	Abcam	According to 1st antibody
Mouse-IgG1 κ, monoclonal	X0931	Dako	According to 1st antibody
2nd antibody for fluorescent IHC			
Donkey-anti-sheep-IgG-AlexaFluor488 (H+L)	713-546-147	Jackson ImmunoResearch Inc	1:800
Donkey-anti-mouse-IgG-AlexaFluor647(H+L)	715-605-150	Jackson ImmunoResearch Inc	1:400
Donkey-anti-rabbit-IgG-Cy3 (H+L)	711-165-152	Jackson ImmunoResearch Inc	1:400
2nd antibody for chromogen IHC	Catalog number	Manufacturer	Applied dilution
Donkey-anti-sheep-IgG-Biotin (H+L)	713-065-147	Jackson ImmunoResearch Inc	1:1000
Goat-anti-mouse-IgG-Biotin (H+L)	115-065-166	Jackson ImmunoResearch Inc	1:800

High throughput sequencing of genetic barcodes

Barcodes were analyzed by deep sequencing as described before (Selich *et al*., 2016). In short, barcodes were amplified by a nested PCR and PCR mixMyFi Mix 23 (Bioline). Individual samples were then labeled by next generation sequencing with adapter-containing index primers. Pooled samples were sequenced by IonTorrent ™ PGM method (Thermo Fisher Scientific). Sequences were then assigned to the samples by a custom Perl 5 script (https://www.perl.org). To remove unspecific amplicons, the sequences were screened for the conserved nucleotides "TACCATCTAGA" and "CTCGAGACT" flanking the barcode region. The last steps of data analysis were performed with customized R scripts (https://www.R-project.org). The histogram was visualized with the R package ggplot2 (Wickham, 2009). Venn diagrams were made with the R package VennDiagram (Chen and Boutros, 2011). Further details are available on request.

Supplemental references

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