Supplementary data

Immunohistochemistry

Paraffin embedded lung tissues were cut into 4 µm thick pieces and mounted on microscope slides. After deparaffinization with decreasing alcohol concentrations the heat induced antigen retrieval was performed with sodium citrate solution (pH=6). Dual enzyme blocking was performed with normal horse serum (DAKO). Six different CD133 antibodies with serial dilutions were used on 4 antigen retrieval systems and 3 developing kits. Additionally, 2 glioblastoma multiforme and prostate cancer tissues were used as positive controls for all antibodies. In order to further characterize the localization of CD133 positive cells, serial sections were made from n=5 IPAH patients and n=5 donors using 3 paraffin blocks from each. 5 slides/block were consecutively stained as follows: CD133, von Willebrand Factor (vWF) and α-smooth muscle actin (α-SMA), Ki67, haematoxilin&eosin (H&E) (Supplementary Figure S3). Negative controls omitted the primary antibody. The primary antibodies against CD133 (host: rabbit), vWF (host:mouse), alpha smooth muscle actin (host:goat) were incubated for 1 hour at room temperature. After washing with PBS, the secondary antibodies were applied and incubated for 1 hour at room temperature. 3,3'-Diaminobenzidine (DAB) was used for antigen detection and the slides were counterstained with methyl green.

In order to further characterize the CD133 positive cells in the lung tissue 2.4 µm thick additional serial sections were made from paraffin embedded lung tissues of n=5 IPAH patients and n=5 donors and were stained as follows: mucin-1 (MUC-1, host:mouse, Novus Biologicals), CD133, surfactant protein C (SPC, host:rabbit, Santa Cruz Biotechnology), vWF+ α-SMA, negative.

Immunofluorescence

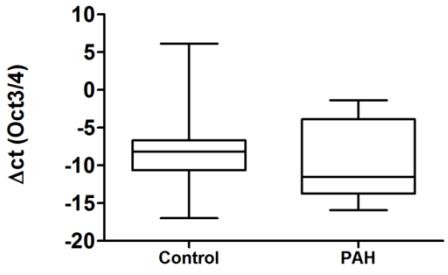
Paraffin embedded tissues were cut into 6 µm thick pieces and mounted on microscope slides. After deparaffinization with decreasing alcohol concentrations the antigen retrieval was performed with trypsin (DAKO). Slides were incubated with CD133 antibody (Abnova, Taipei, Taiwan) for 30 minutes. After fixation with 1.4% paraformaldehyde the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) containing medium. The slides were analyzed with a laser-scanning confocal microscope, LSM 510 Meta (Zeiss), with the following Ex/Em settings: 405/BP420-480 (DAPI); 488/BP505-550 (Alexa Fluor 488) and 633/679-754 (Alexa Fluor 680). The 1024x1024 resolution images were taken with a Zeiss 40x oil immersion objective with 1.4 NA.

In order to further characterize the CD133 positive cells in the lung tissue double stainings were performed using 4 µm thick pieces which were mounted on microscope slides. Lung tissues of n=5 IPAH patients and n=5 donors were stained as follows: After deparaffinization with decreasing alcohol concentrations the antigen retrieval was performed with Trypsin for 15 minutes. The slides were incubated with the primary antibodies (CD133 and MUC-1) for 2 hours. Fluorescence tagged secondary antibodies were then applied (Alexa Fluor 488 and Alexa Fluor 555, Invitrogen) and the slides were incubated for 1 hour. After fixation with 1.4% paraformaldehyde the slides were mounted with 4',6-diamidino-2-phenylindole (DAPI) containing medium and analysed as described above.

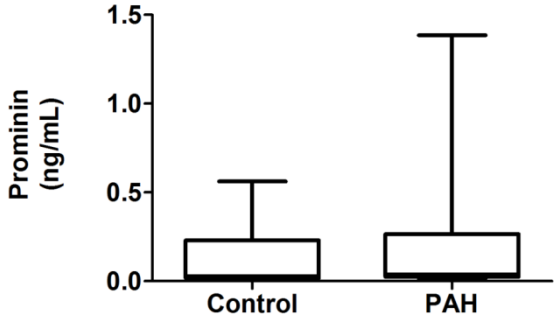
RT-PCR

Blood was taken from 10 healthy subjects and 10 PAH patients and the CD133 positive cells were sorted using MACS (AutoMACS, Milteny, Vienna, Austria). Total RNA was extracted from the isolated cells using peqGOLD Total RNA Kit (Peqlab). A preamplification kit was used for cDNA synthesis (NuGEN, Ovation PicoSL System V2, Berlin, Germany). mRNA levels of octamer binding transcription factor (Oct3/4), sex determining region Y-box 2 (SOX2), homeobox transcription factor (Nanog), C-X-C chemokine receptor type 4 (CXCR4) and Ki67 were assessed by RT-PCR on a Light Cycler 480 (Roche). Primers were designed on intron spanning sequences (Supplementary Material Table S4). Beta-2 macroglobulin was used as a housekeeping gene. For mRNA expression the delta CT method was used.

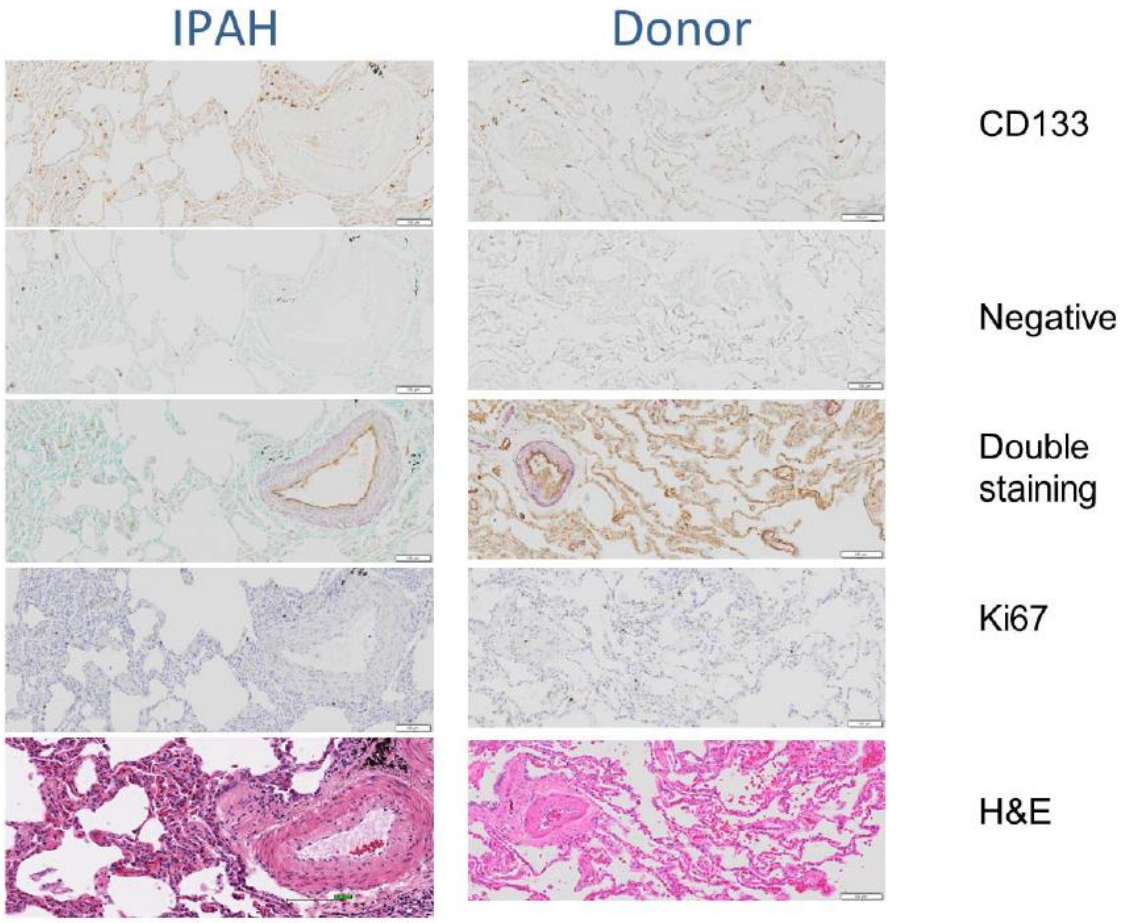
Supplementary Figure S1. Real time PCR analysis of Oct3/4 from isolated CD133 cells in N=10 donor vs. n=10 PAH patients.



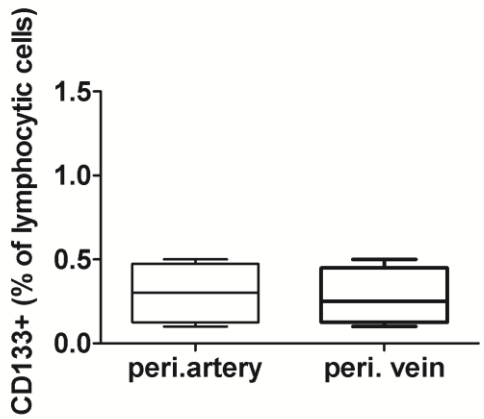
Supplementary Figure S2. Plasma levels of CD133 (prominin-1) in 20 PAH patients and their age- and sex-matched controls as determined by ELISA. Mann Whitney U test was used for the statistical analysis.



Supplementary Figure S3



Supplementary Figure S4. Flow cytometric analysis of CD133 positive cells using blood from peripheral artery (peri. artery) and peripheral vein (peri. vein) of four PAH patients.



Supplementary Figure S5

A representative image of the double immunofluorescent staining for CD133 (green) and MUC1 (red) focusing on a bronchus with an inset on bronchiolar epithelial cells. The white arrow indicates a double positive bronchiolar epithelial cell. **B**. representative image of a double immunofluorescent staining for CD133 and MUC1 in donor lung. **C** representative image of a double immunofluorescent staining for CD133 and MUC1 in IPAH lung.

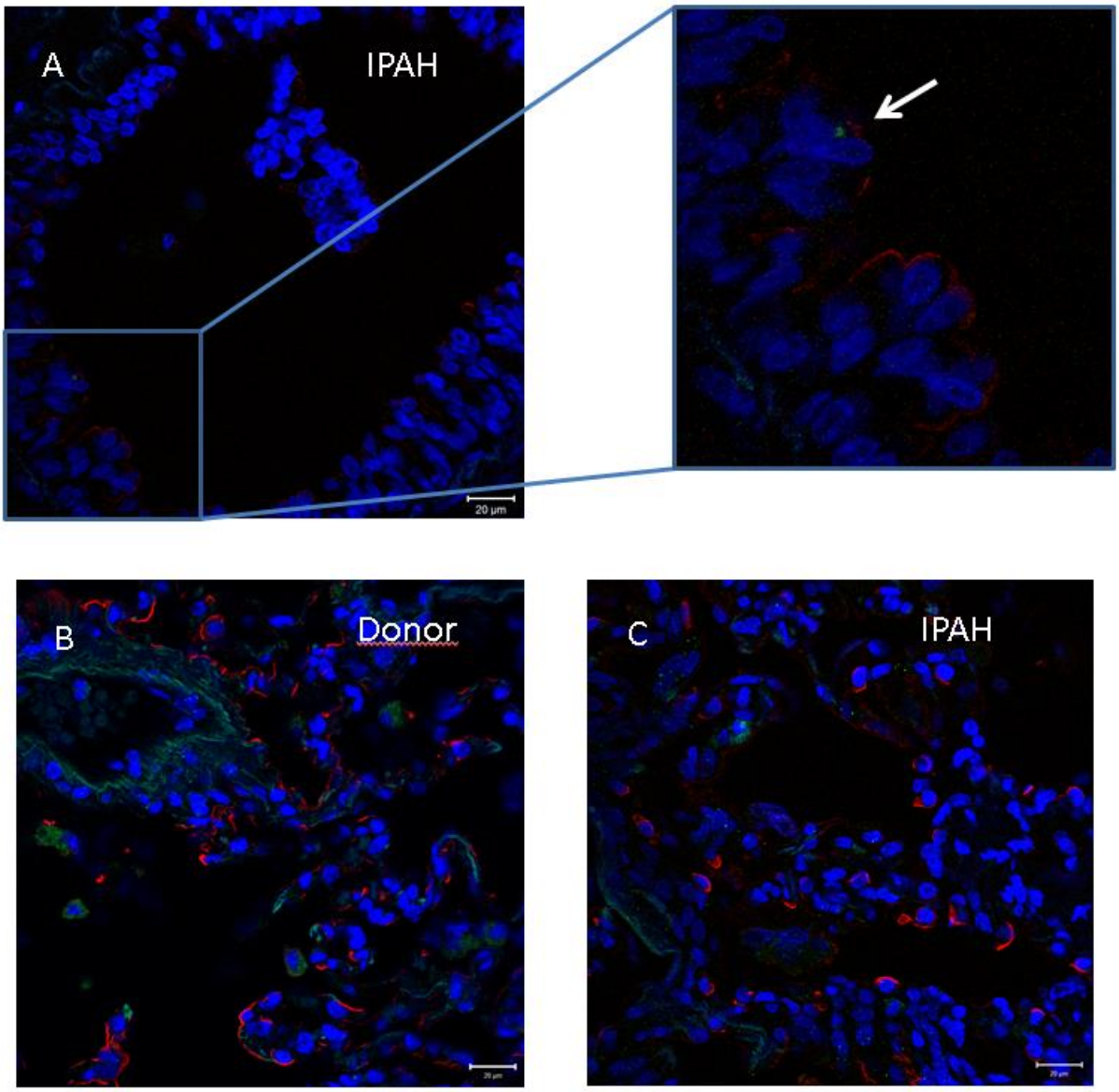


Table S1

Antibody-fluorochrome conjugations

Antibody	Fluorochrome	Isotype Control	Clone	Company
CD117	PE-Cy7	Mouse IgG1, k	104D2	BioLegend
CXCR2	PerCP	Mouse IgG_{2A}	48311	R&D Systems
CD309	PE	Mouse IgG1	89106	R&D Systems
CD34	AF488	Mouse IgG1, k	581	BioLegend
CD14	APC-eFluor 780	Mouse IgG1, k	61D3	eBioscience
CD31	AF700	Mouse IgG1	MEM-05	eBioscience
CD133	APC	Mouse IgG12b	293C3	Milteny Biotech
CD16	BD Horizon V500	Mouse IgG1, k	3G8	BD Horizon
CD45	eFluor 450	Mouse IgG1, k	HI30	eBioscience

Table S2

Instrument configuration

Laser			PMT	Dichroic Mirror/	Bandpass filter (nm)	Fluorochrome
Wavelength (nm)	Power (mW)	Type		Longpass Filter (nm)		
Argon 488			A	735	780/60	PE-Cy7
			C	635	670/14	PerCP
			E	550	576/26	PE
			F	505	530/30	AF488
Red Diode 633			A	755	780/60	APC-eFluor780
			B	675	730/45	AF700
			C	N/A	660/20	APC
Violet 405			A	505	525/50	BD V500
			B	N/A	440/40	eFluor450

Table S3

Compensation Matrix

Fluorochrome	(-)% Fluorochrome	Spectral Overlap
PE	AF488	17
APC	AF488	1
AF488	PE	1
PECy7	PerCp	12
APC	PerCp	7
PE	PE-Cy7	1
PerCp	PE-Cy7	2
APC-Cy7	PE-Cy7	6
AF700	APC	23
APC-Cy7	APC	5,8
APC	AF700	1
APC-Cy7	AF700	14
AF488	AmCyan	3,1
AmCyan	Pacific Blue	9
AF700	APC-Cy7	12
APC	APC-Cy7	8
PE-Cy7	APC-Cy7	10

Table S4

Primer list

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
Oct 3/4	CTGGGGGTTCTATTTGGGAAGG	GTTCGCTTTCTCTTTCGGGC	194
SOX2	GCGTCAAGCGGCCCA	GCTTCTCCGTCTCCGACAAA	144
Nanog	ATGCCTCACACGGAGACTGT	AGGGCTGTCCTGAATAAGCA	65
CXCR4	CCAGTAGCCACCGCATCT	ATAGTCCCCTGAGCCCATTT	99
Ki67	ACGAGACGCCTGGTTACTATC	GCTCATCAATAACAGACCCATTTAC	225
β2-	CCTGGAGGCTATCCAGCGTACTCC	TGTCGGATGGATGAAACCCAGACA	112

macroglobulin			