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Supplemental Information

miR-150-PTPMT1-cardiolipin signaling in pulmonary arterial hypertension

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SUPPLEMENTAL MATERIAL

"Mir-150-PTPMT1-Cardiolipin signaling in Pulmonary Arterial Hypertension"

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Animal experiments

All studies were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986. All animals were randomly allocated to groups, and all personnel involved in data collection and analysis (haemodynamics and histopathologic measurements) were blinded to the treatment status of each animal. Only weight-and age-matched males were included for experimentation as, in contrast to the human clinical studies, most animal studies have shown that female sex and estrogen supplementation have a protective effect against PAH.

To induce PAH, 8-12 weeks old C57/BL male mice (20 g; Charles River, UK) were injected subcutaneously with Sugen (SU5416; 20mg/kg), suspended in 0.5% [w/v] carboxymethylcellulose sodium, 0.9% [w/v] sodium chloride, 0.4% [v/v] polysorbate 80, 0.9% [v/v] benzyl alcohol in deionized water once/week. Control mice received only vehicle. Mice were either housed in normal air or placed in a normobaric hypoxic chamber (10% O_2) for 3 weeks (n= 8/group).

mirVana hsa-miR-150-5p (ID MC10070) mimic or scrambled miRNA control (Ambion) in complex with DACC lipoplex preparation (Silence Therapeutics)[1] was administered intravenously via tail vein injection once every fourth day at 1.5 mg/kg/day for 3 weeks, on 5 occasions. The first injection was given 1 day before Sugen/hypoxia administration. At 3 weeks, the mice were anaesthetised by intraperitoneal injection of Ketamine/Dormitor (75 mg/kg + 1 mg/kg).

In addition, to follow the distribution of liposomal cargo, a single intravenous injection of fluorescently labelled DACC/siRNA-Cy3 (2.8 mg/kg body weight) in healthy mice was performed. Localization of the fluorescently-labelled RNA in paraffin sections of lung, heart, kidney, spleen and liver taken 4 and 24 hours post-injection, was analysed with fluorescent confocal imaging.

To produce mice with inducible conditional endothelium-specific deletion of miR-150, floxed miR-150 mice (STOCK Mir150^{tm1Mtm}/Mmjax mice from Jackson Laboratories) C57/Bl6 background were crossed with C57/Bl6 mice carrying tamoxifen-inducible Cre recombinase under the control of the Cdh5 promoter (Cdh5(PAC)-iCreERT2[2]. Following tamoxifen administration, efficient Cre-recombinase deletion of miR-150 was confirmed by PCR in miR-150$^{fl/fl}$/Cdh5(PAC)-iCreERT2 mice (henceforth referred to as miR-150iEC-KO).

Weaned mice were ear-notched and samples were incubated in lysis buffer (100 mM Tris HCl pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, 0.14 mg/mL Proteinase K, Ambion^{TM}) for 2 hours at 55°C under agitation (700 rpm). Samples were then vortexed and pelleted at 14,000 rpm for 10 minutes. Supernatant was transferred to a new DNase-free tube and DNA was precipitated in isopropanol (20 minutes incubation at RT). DNA was pelleted at 14,000 x g for 10 minutes, supernatant was discarded and the DNA pellet was air dried and then resuspended in 100 µL of DNase-free water.

PCR reactions were performed using REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, cat. R2523) with the primers listed in Supplemenrary Table S1 (500 nM of each) in a SmplyAmp^{TM} Thermal Cycler (Applied Biosystems). All primers were purchased from Sigma-Aldrich.

Thermocycling conditions for miR-150 followed the Jackson Laboratory's instructions (www.jax.org): 2 min at 94°C, then 20 s at 94°C, 15 s at 65°C, 10 s at 68°C, for 10 cycles, 15

s at 94°C, 15 s at 60°C, 10 s at 72°C, for 28 cycles, and a last step of 2 min at 72°C. For Cre genotyping, thermocycling conditions were as follows: 3 min at 94°C, then 30 s at 94°C, 30 s at 70°C, 60 s at 72°C, for 32 cycles, and a last step of 10 min at 72°C.

All PCR products were separated on a 2% agarose gel, visualized using GelRed Nucleic Acid Gel Stain (Thermo Fisher Scientific, cat. NC0017761), and size was estimated with comparison to a DNA mass ladder (GeneRuler 100 bp DNA Ladder, Thermo Fisher Scientific, cat. SM0243).

At 6 weeks of age, miR-150^{fl}/Cdh5(PAC)-iCreERT2 mice were injected intraperitoneally with 100 µL of 5 mg/mL tamoxifen (Sigma, cat. no. T5648) or vehicle (12.5% vol/vol ethanol in peanut oil) for 5 consecutive days[3]. Littermate wild-type animals were used as control. Two weeks after tamoxifen injection, mice were injected with Sugen and housed in normal air or hypoxia for 3 weeks (n=4-8/group).

The development of PAH was verified by measuring right ventricular systolic pressure (RVSP), right ventricular hypertrophy (assessed as the right ventricle to left ventricle/septum ratio - RV/LV+S), and muscularisation of small intrapulmonary arteries, as previously described[4]. RVSP was measured via direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetized animal. Pressure measurements were repeated three times and the mean value used. Data were collected by Power Lab Data Acquisition system (AD Instruments) and analysed using LabChart 8 software (AD Instruments) by an investigator blinded to the treatment group.

The right lung lobe was harvested and snap frozen in liquid nitrogen or placed in RNAlater® RNA Stabilization Solution for RNA isolation. The left lobe was inflation-fixed (10% formaldehyde in PBS), embedded in paraffin, and sectioned for histology. The heart and liver were collected and snap frozen or placed in RNAlater®. Transverse formalin-fixed lung sections

were stained with an anti-smooth muscle actin antibody (DAKO M0851) or Verhoeff's van Gieson stain (EVG) to visualise elastic lamina. Pulmonary vascular remodelling (muscularisation of small intrapulmonary arteries) was determined by counting all muscularised vessels with a diameter smaller than 50 μm in each lung section and expressed as a % of all (muscularised + non-muscularised) vessels. Counting was performed by two observers blinded to treatment.

In situ hybridization

In situ hybridization was carried out on paraffin lung sections of untreated mice and Sugen/hypoxia mice (n=3, 5 weeks hypoxia) using miRCURY LNA™ microRNA ISH Optimization kit (Exiqon, cat no 339459). Negative control: LNA™ scrambled microRNA probe, double DIG labelled (40 nM); Positive controls: LNA™ U6snRNA probe, 5'DIG-labeled (1 nM), LNA™ microRNA223 probe, double DIG labelled (40 nM, labels myeloid, granulocytic and monocytic cell lineages in the hematopoietic system). LNA™ microRNA150 probe double DIG labelled (40 nM) was used to study changes in miR-150 levels. Hybridization temperature: 54°C. The sections were incubated with sheep anti-DIG antibody (1:200, Roche Applied Science; cat. no 1333 089), biotinylated donkey anti-sheep antibody (1:200, Sigma, cat no. AP184B), streptavidin-peroxidase conjugate (1:200), followed by DAB/hematoxylin staining. A detailed protocol can be found in miRCURY LNA miRNA Detection Probes Handbook – Qiagen.

RNAscope®

For formalin-fixed, paraffin-embedded lung sections, RNAscope® Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics) and TSATM Cyanine 3 & 5, TMR, Fluorescein Evaluation Kit System (PerkinElmer) were used according to manufactures' protocols[5].

Briefly, tissue sections in 5-µm thickness were baked in a dry oven (Agilent G2545A Hybridization Oven, Agilent Technologies) for 1 hour at 60°C, and deparaffinised in xylene, followed by dehydration in 100% ethanol. Tissue sections were then incubated with RNAscope® Hydrogen Peroxide for 10 minutes at room temperature. After washing twice with distilled water, manual target retrieval was performed boiling the sections (100°C to 103°C) in 1X Target Retrieval Reagents using a hot plate for 15 minutes. Slides were then rinsed in deionized water, 100% ethanol, and incubated with RNAscope® Protease Plus at 40°C for 30 minutes in a HybEZ hybridization oven (Advanced Cell Diagnostics, PN 321710/321720). Hybridization with target probes (Mm-Myb-C1, NM_001198914.1; Mm-Notch3-C2, NM_025576.2) was carried out incubating the slides at 40°C for 2 hours. Two different probes/channels (C1-C2) were multiplexed. After washing twice with Wash Buffer, slides were stored overnight in 5x SSC buffer (0.75M NaCl, 0.075M sodium citrate). The following day, the slides were incubated at 40°C with the following reagents: Amplifier 1 (30 min), Amplifier 2 (30 min), Amplifier 3 (15 min); HRP-C1 (15 min), TSA® Plus fluorophore for channel 1 (fluorescein, PerkinElmer; 1:1000; 30 min), HRP blocker (15 min); HRP-C2 (15 min), TSA® Plus fluorophore for channel 2 (cyanine 3, PerkinElmer; 1:1000; 30 min), HRP blocker (15 min). After each hybridization step, slides were washed three times with Wash Buffer at room temperature.

RNAscope hybridisation was combined with immunofluorescence[5, 6]. Tissue was blocked for 1 hour at room temperature with 3% normal horse serum (Vector Laboratories) in 1X PBS containing 0.1% bovine serum albumin (Sigma-Aldrich), and 0.01% sodium azide (Sigma-Aldrich), and then incubated with polyclonal rabbit antibody raised against human von

Willebrand Factor (1:500; A0082, Dako), at 4°C overnight. After three washes in PBS, slides were incubated with FITC-labelled Goat Anti-Rabbit antibody (1:100; 111-095-003, Jackson ImmunoResearch Inc.) for 30 minutes at RT. Following immunostaining, tissues were mounted in Vectashield with DAPI and examined under a fluorescent confocal microscope (Leica, TCS SP5, Leica Biosystems, Bretton, Peterborough).

Cell culture

Human pulmonary artery endothelial cells (HPAECs, Promocell, C-12241) were cultured in endothelial cell growth medium 2 (ECGM2; PromoCell, C-22111) and human pulmonary artery smooth muscle cells (HPASMCs, Lonza, CC-2581) in smooth muscle cell growth medium 2 (SMCGM2, PromoCell, C-22062), as previously described [4]. In some experiments, the cells were exposed to hypoxia (5% CO_2, 2% O_2) for 18-72 hours.

For non-contact co-culture of HPAECs and HPASMCs, Transwell dishes with 0.4 μm pore polyester membrane inserts (Scientific Laboratory Supplies, UK) were used. HPAECs were seeded into the fibronectin-coated top chambers and cultured in complete ECGM2 medium, whereas HPASMCs were seeded at the bottom of the plate and cultured in complete SMCGM2 for 24 h. The two cell types were then washed with PBS, combined together and co-cultured in endothelial cell basal medium supplemented with 10% FBS (Sigma-Aldrich, F7524), and selected components of ECGM2 supplement pack (PromoCell, C-22111): EGF (2.5 ng/L), FGF (10 ng/L), IGF (20 ng/L) with 1% penicillin and streptomycin.

Blood-derived human endothelial cells and human lung samples

All investigations were conducted in accordance with the Declaration of Helsinki. Venous blood samples were obtained with the approval of the Brompton Harefield & NHLI and

Hammersmith Hospitals Research Ethics committees and informed written consent from healthy volunteers (n=14) and patients with idiopathic PAH (IPAH, n=12). Participants were identified by number. Human endothelial colony forming cells (ECFCs) were derived from peripheral blood samples as previously described [4]. Clinical information is shown in Table S2.

Cell Transfection

Briefly, HPAECs were left untreated (control) or were transfected with control miRNA (non-targeting transfection control; Ambion Life Technologies, 4464076) at 20 nmol/L, or miRVana^{TM} has-miR-150-5p, (4464066 Assay ID MC10070;), miRVana^{TM} miRNA inhibitor, (4464084, Assay ID MH10070), both at 20 nmol/L, or control siRNA (non-targeting negative control siRNA; Invitrogen, 4390843) at 10 nmol/L, or siPTPMT1 (4392420 Assay ID s229946) at 10 nmol/L, using Lipofectamine RNAiMAX in antibiotic-free media, following manufacturer's instruction. After 24 hours, the media were changed and cells were exposed to hypoxia for 24-72 hours. Alternatively, on the following day, the untransfected and transfected cells were starved for 9 hours before caspase 3/7 assay. Human pcDNA PTPMT1, NM_175732.2 (clone OHu11042; 2B Scientific Ltd. Upper Heyford, UK) was transfected into HPAECs with Lipofectamine RNAiMAX at 2ng/well in a 24-well dish, as recommended by the manuafacturer. Transfection efficiency was measured by the uptake of Cy3™ Dye-Labeled Pre-miR Negative Control (AM17120; Thermo Fisher Scientific) and quantitative real-time PCR (RT-qPCR). All experiments were performed 24 hours after transfection. Transfected cells were exposed to hypoxia (2% O_2, 5% CO_2), serum and growth factor depletion or inflammatory cytokines. Cell proliferation and NFκB activity assays were carried out 72 and 48 hours post-transfection, respectively.

RNA Extraction

RNA was extracted from cultured cells or tissue (~10 mg) stored in RNALater® using Monarch® Total RNA Miniprep Kit (New England BioLabs). For maximal RNA recovery, tissues was mechanically homogenized using a Kinematica™ Polytron™ PT 1300 D and incubated at 55°C for 5 minutes with Proteinase K following manufacturer's instructions. To remove any residual DNA that may affect downstream applications, an On-Column DNase I digestion was also performed. RNA concentration and purity was evaluated using NanoDrop 2000 spectrophotometer (Thermo Scientific). The A260/230 and A260/280 ratios were used to assess the presence of contaminants. RNA was then stored at -80°C for later experiments.

Real-time quantitative PCR

Input RNA (50-100 ng/µL) was reverse-transcribed using LunaScript® RT SuperMix Kit (New England BioLabs) or TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and custom Multiplex RT Primer pool in a SimpliAmp™ Thermal Cycler (Applied Biosystems), according to the manufacturer's instructions. The multiplex RT primer pool consisted of primers for miR-150-5p and U6 (Thermo Fisher Scientific). No-template samples where included as negative controls.

TaqMan® miRNA Assays for hsa-miR-150-5p (Assay ID 000473), and U6 snRNA (Assay ID 001973), and TaqMan® Gene Expression Assays for PTPMT1 (Hs00378514_m1, Mm00458631_m1), SERPINE1 (Hs00167155_m1), PERP (Hs00953482_g1), DUSP5 (Hs00244839_m1), c-MYB (Hs00920556_m1, Mm00501741_m1), NOTCH3 (Hs01128537_m1, Mm01345646_m1), Col1a1 (Mm00801666_g1), Rcan1 (Mm01213406_m1), Tgfb1 (Mm01178820_m1), and GAPDH (Hs02786624_g1, Mm99999915_g1; all Thermo Fisher Scientific), were used to perform quantitative PCR

(qPCR). No-template samples were included as negative controls and all PCRs were performed in triplicate. The reaction was performed on a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Data were analysed using QuantStudio 12K Flex Software version 1.2 (Applied Biosystems). For relative quantification, the data were analysed using the $2^{-\Delta\Delta Ct}$ method, where U6 snRNA and GAPDH were used as endogenous normalization controls for miR-150 and gene expression, respectively.

RNA-Sequencing and identification of signaling mediators of miR-150

10µl of RNA (250-300 ng/µl) extracted from cells transfected with miR-150 mimic or scrambled control in three independent experiments, were sent in to Imperial BRC Genomics Facility (Imperial College of London, UK) for next-generation RNA-sequencing RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a Qubit 4 Fluorometer (Thermo Fisher Scientific). RNA libraries were prepared using TruSeq® Stranded mRNA HT Sample Prep Kit (Illumina Inc., USA) according to the manufacturer's protocol as previously described[6]. Libraries were run over 4 lanes (2 x 100 bp) on a HiSeq 2500 (Illumina Inc.) resulting in an average of 34.4 million reads per sample. Sequence data was de-multiplexed using bcl2fastq2 Conversion Software v2.18 (Illumina Inc.) and quality analysed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Transcripts from paired-end stranded RNA-Seq data were quantified with Salmon v 0.8.2 using hg38 reference transcripts[7, 8]. Count data was normalised to accommodate known batch effects and library size using DESeq2[9]. Pairwise differential expression analysis was performed based on a model using the negative binomial distribution and p-values were adjusted for multiple test correction using the Benjamini-Hochberg procedure[10]. Genes were considered differentially expressed if the adjusted p-value was greater than 0.05 and there was at least a 1.5 fold change in expression. miRNA target prediction was carried out with TargetScan Human, miRecords and Ingenuity

Expert Findings. Gene enrichment was carried out using Ingenuity Pathway Analysis (IPA, Qiagen).

Accession numbers for RNA sequencing data will be provided upon provisional acceptance of the manuscript.

Caspase 3/7 apoptosis assay

Cells were incubated in serum- and growth factor-depleted medium for 9 hours to induce apoptosis. Apoptosis was measured using Cell Meter^{TM} Caspase 3/7 Activity Apoptosis Assay Kit (AAT Bioquest, ABD-22796). Fluorescence intensity was analysed in Glomax^{TM} luminometer at Ex/Em = 490/525 nm.

NFκB luciferase reporter assay

NFκB activity was measured in luciferase reporter assay [4] in the Glomax^{TM} luminometer.

EdU Proliferation Assay

The EdU Cell Proliferation Assay Kit (EdU-594, EMD Millipore Corp, USA, 17-10527) was used to measure cell proliferation, according to the manufacturer's protocol. Proliferation of ECFCs from healthy individuals and IPAH patients was evaluated in serum-reduced, growth factor-depleted media.

Seahorse Bioenergetics Assay

Oxygen consumption (OCR) and extracellular acidification rates (ECAR) were measured in Seahorse Extracellular Flux Analyzer using XF24 (Seahorse Bioscience, North Billerica, MA) and Seahorse XF Mito Stress Test Kit (Agilent, 103015-100). 4 x 10^4 cells were plated into each well prior to the assay. Cells cultured on the Seahorse XF Cell Culture microplates were left untreated or were transfected with miR-150 or PTPMT1, as previously described for 24h overexpression. The sensor cartridge was hydrated at 37°C in Seahorse XF Calibrant overnight in a non-CO_2 incubator.

The assay medium was prepared by supplementing Seahorse XF Base Medium with 1mM pyruvate (S8636), 2mM glutamate and glucose (G8540) and 10mM glucose (G8769), warming it up to 37°C and adjusting pH to 7.4. All compounds were warmed up to room temperature. 1μM oligomycin, 1μM FCCP and 0.5μM rotenone/antimycin A provided with the kit were loaded into the appropriate ports of hydrated sensor cartridge. The cells were incubated with assay medium for 1h before Seahorse XF Mito Stress Test. OCR and ECAR were normalized to the protein concentration.

Immunostaining

Immunostaining of paraffin embedded lung sections was carried out as previously described[4].

To stain mitochondria, HPAECs cultured on Thermanox® Plastic Coverslips (13 mm) were washed twice in PBS, fixed in 4% formaldehyde for 15 min at room temperature, washed in PBS and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich, 234729) in PBS for 10 min. The cells were then rinsed with PBS, blocked in 10% normal goat serum (Vector Laboratories, S-1000) for 1 h and incubated with mouse anti-mitochondria antibodies (Abcam, ab92824) diluted 1:100 in PBS in 5% BSA in a humidified chamber overnight. Cells were then rinsed 3 times with PBS and incubated with FITC-Goat Anti-Mouse IgG (Jackson ImmunoResearch

Inc.,115-095-003; 1:200) with tetramethylrhodamine (TRITC)-phalloidin (1 μg/mL; Sigma-Aldrich, UK, P1951) for 1h. Following immunostaining, cells were mounted in Vectashield Antifade Mounting Medium containing nuclear stain DAPI (Vector Laboratories, H-1200) and examined under the fluorescent confocal microscope (Leica, TCS SP5, Leica Biosystems, Bretton, Peterborough).

Cardiolipin measurement

Quantification of cardiolipin in cells and tissues was carried out with Cardiolipin Assay Kit (BioVision, cat. K944-100), according to the manufacturer's instructions.

Mitochondrial fragmentation count and mitochondrial content

Mitochondrial were immunolabelled, as described above. Mitochondrial fragmentation (area taken by mitochondrial particles < 2μm in length)[11] and total mitochondrial coverage (area taken by all mitochondria) were determined using NIP2 image software [12] . The 2μm cut-off size was optimal[11] in selection of mitochondria unassociated with mitochondrial network. Briefly, the acquired images were filtered (median), thresholded, and binarized to identify individual mitochondrial segments and score the total area of fragmented mitochondria. This value was normalized to the total mitochondrial area (in pixels) in each cell, to define the individual cell's MFC. For each intervention 20 randomly selected cells were analysed in 3 separate experiments [13].

Table S1. Sequences of specific primers used for mouse genotyping.

Gene	Primer	Sequence	Amplicon length (bp)

MIR150	Forward	5’-GTTCAAGCAGATCATGATACTCAA-3’	304 (WT) - 396 (Mutant)
	Reverse	5’-GTCCTGGGACAGAGCAAAGATT-3’	
Cre	Forward	5’-GCCTGCATTACCGGTCGATGCAACGA-3’	720 (Mutant)
	Reverse	5’-GTGGCAGATGGCGCGGCAACACCATT-3’	

Table S2. Demographic and clinical features of patients and healthy volunteers. Venous blood samples were obtained with local ethics committee approval and informed written consent from healthy volunteers and patients with idiopathic PAH (IPAH). Data represented as median (range).

		Control (n=14)	IPAH (n=12)
Male/Females		3/11	1/11
Age (years)		30.0 (23.0 - 45.0)	38.33 (27.0 - 67.0)
Time from diagnosis (months)		-	13.0 (0.1 - 60.0)
mPAP (mmHg)		-	65.0 (28.0 - 94.0)
Six minute walk distance (m)		-	396.0 (300.0 – 540.0)
WHO Functional Class	I	-	1
	II	-	3
	III	-	5
	IV	-	3
Treatment naïve		-	2
Warfarin		-	4
Calcium Antagonists		-	1
ER Antagonists		-	7
PDE5 Inhibitors		-	8
Prostanoids		-	4
Statins		-	0

mPAP, mean Pulmonary Arterial Pressure; ER, Estrogen-Receptor; PDE5, Phosphodiesterase type 5.

Table S3. Top 26 differentially expressed genes (DEG, adjusted p-value<0.05) in miR-150 transfected HPAECs (RNA-Sequencing).

Gene ID	Log_2FC	Adj p-value	Entrez Gene Name	Location	Type(s)
PTPMT1	1.329	5.48×10^{-7}	protein tyrosine phosphatase, mitochondrial 1	Cytoplasm	phosphatase
PERP	-0.904	8.88×10^{-6}	PERP, TP53 apoptosis effector	Plasma Membrane	other
H3F3A	0.698	8.92×10^{-6}	H3 histone family member 3A	Nucleus	other
DDX3Y	0.699	9.52×10^{-5}	DEAD-box helicase 3, Y-linked	Cytoplasm	enzyme
AP2A2	6.084	2.54×10^{-4}	adaptor related protein complex 2 alpha 2 subunit	Cytoplasm	transporter
IFIT2	-0.616	2.54×10^{-4}	interferon induced protein with tetratricopeptide repeats 2	Cytoplasm	other
EHMT2	2.405	2.00×10^{-3}	euchromatic histone lysine methyltransferase 2	Nucleus	transcription regulator
SERPINE1	-0.430	2.13×10^{-3}	serpin family E member 1	Extracellular Space	other
SNCA	0.686	3.24×10^{-3}	synuclein alpha	Cytoplasm	enzyme
ANKRD28	-0.620	7.30×10^{-3}	ankyrin repeat domain 28	Cytoplasm	other
IRAK2	-0.522	7.31×10^{-3}	interleukin 1 receptor associated kinase 2	Plasma Membrane	kinase
MLH1	0.773	7.31×10^{-3}	mutL homolog 1	Nucleus	enzyme
KIDINS220	-0.627	7.42×10^{-3}	kinase D interacting substrate 220	Nucleus	transcription regulator
MAMDC2	-0.613	9.04×10^{-3}	MAM domain containing 2	Extracellular Space	other
ZNF500	0.986	0.0156	zinc finger protein 500	Nucleus	other
DUSP5	-0.572	0.0179	dual specificity phosphatase 5	Nucleus	phosphatase
SLC2A10	2.272	0.0195	solute carrier family 2 member 10	Plasma Membrane	transporter
JAG1	0.457	0.0214	jagged 1	Extracellular Space	growth factor
CAPN5	0.917	0.0284	calpain 5	Cytoplasm	peptidase
SRPRA	0.468	0.0329	SRP receptor alpha subunit	Cytoplasm	other
KCTD20	-0.611	0.0349	potassium channel tetramerization domain containing 20	Cytoplasm	other
SEMA3A	1.637	0.0366	semaphorin 3A	Extracellular Space	other
SOD2	-0.392	0.0369	superoxide dismutase 2	Cytoplasm	enzyme
BOD1	0.624	0.0377	biorientation of chromosomes in cell division 1	Nucleus	other
MET	0.522	0.0378	MET proto-oncogene, receptor tyrosine kinase	Plasma Membrane	kinase
GGT1	1.759	0.0379	gamma-glutamyltransferase 1	Plasma Membrane	enzyme

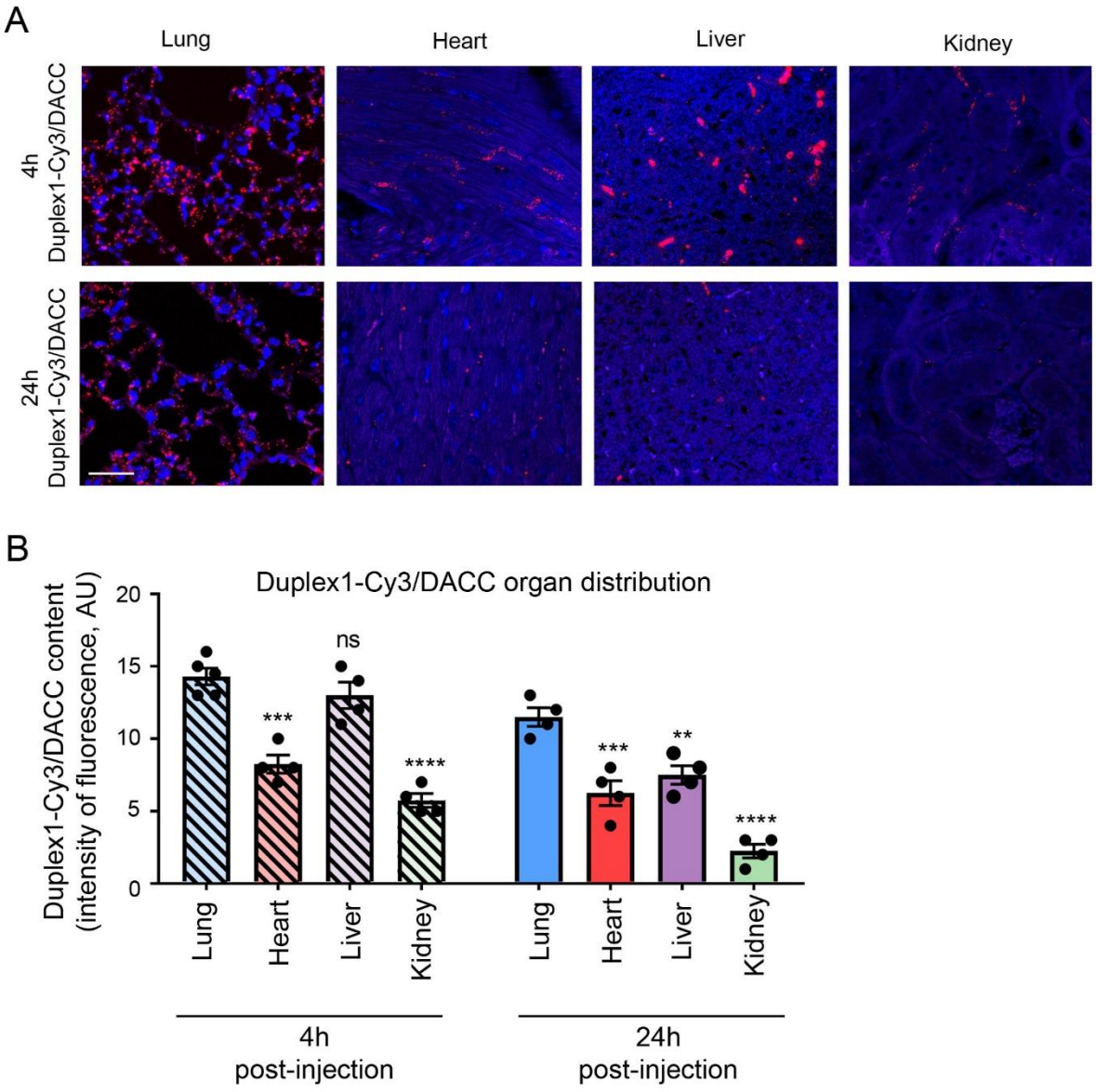

Figure S1. Distribution of fluorescent RNA marker delivered in DACC liposomes in mouse lung, heart, liver and kidney. siRNA-Cy3/DACC (Duplex1-Cy3/DACC) or (vehicle only) were delivered to mice by iv injection and tissue distribution of fluorescent siRNA was studied 4 hr or 24hr later. (A) Representative images and (B) a corresponding graph showing distribution of Duplex1-Cy3/DACC in different organs, as indicated. In (A) nuclei are blue (DAPI), while siRNACy3 is red. Bar=25 μm. In (B) **p<0.01, ***p<0.001, ****p<0.0001, comparisons with 24h lung (for 24h group) or 4h lung (for 4h group), as appropriate; N=4-5.

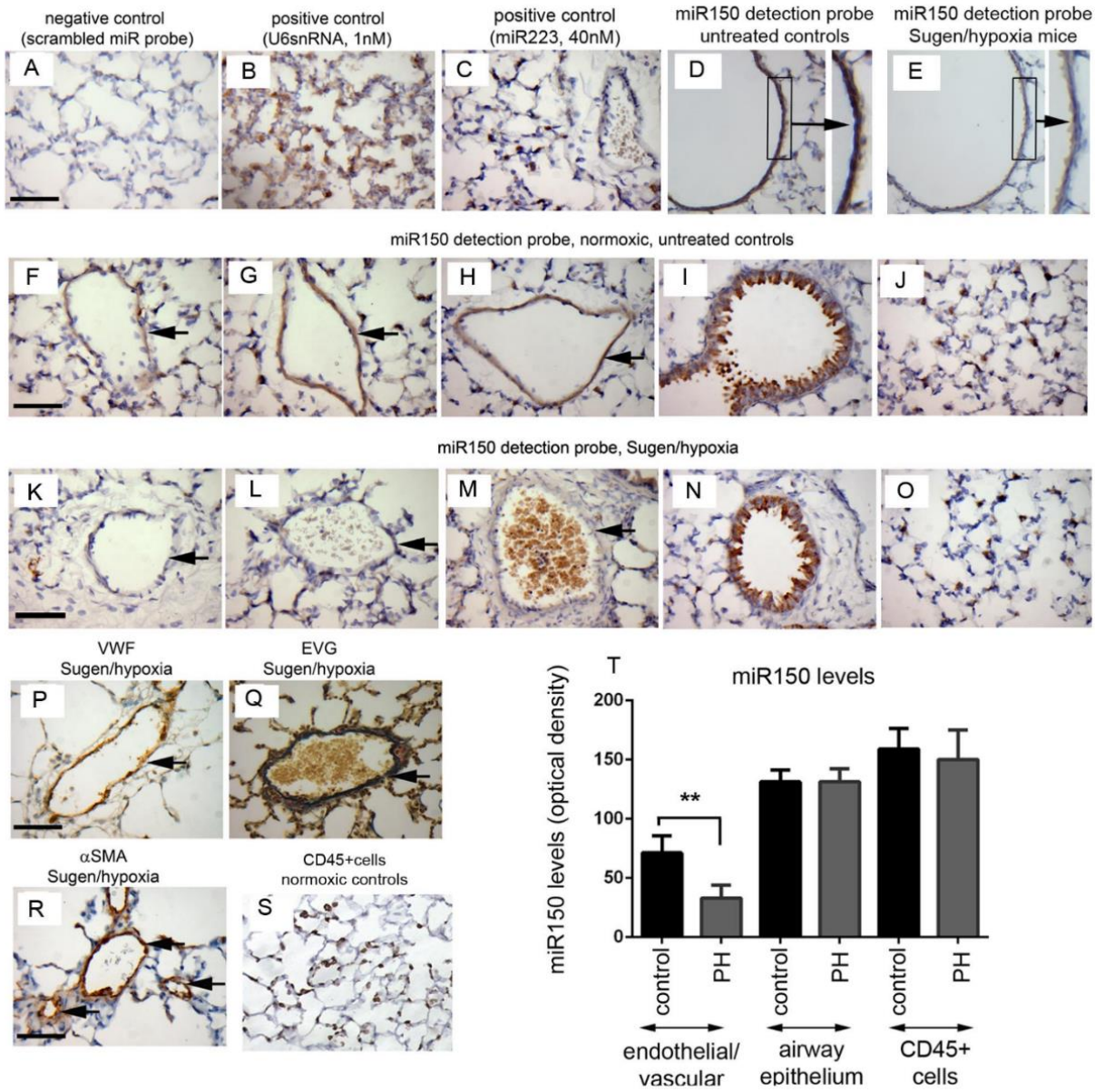

Figure S2. **miR-150 levels are decreased in the endothelium of pulmonary hypertensive hypoxia/Sugen mice; in situ hybridization.** (A-C) negative and positive controls; (D, E) miR-150 in pulmonary vascular endothelium in control mice and Sugen/hypoxia mice, as indicated; (F-H) miR-150 staining in the small intrapulmonary vessels (arrows) in control lungs; (I and J) corresponding miR-150 staining in the airway epithelium and leukocytes, respectively. (K-M) miR-150 staining in intrapulmonary vessels of Sugen/hypoxia mice; (N and O) corresponding miR-150 staining of the airway epithelium and leukocytes in Sugen/hypoxia lungs, respectively. (P, Q and R show vWF, EVG and αSMA staining of the remodelled intrapulmonary vessels in Sugen/hypoxia mice. (S) localization of leukocytes (CD45+cells) in control lung; Bar=40μm. The graph shows relative changes in miR-150 levels in endothelial/vascular tissues, airway epithelium and $CD45^+$ cells in lung tissues from control and PH mice (3 mice/group, 10 vessels/mouse). Optical density of selected 7-10 regions of interest within the endothelial layer or 10 $CD45^+$ cells was measured with Image Pro Plus software. Bars are means ± SEM. *$p<0.05$, comparison with control. n=5/group

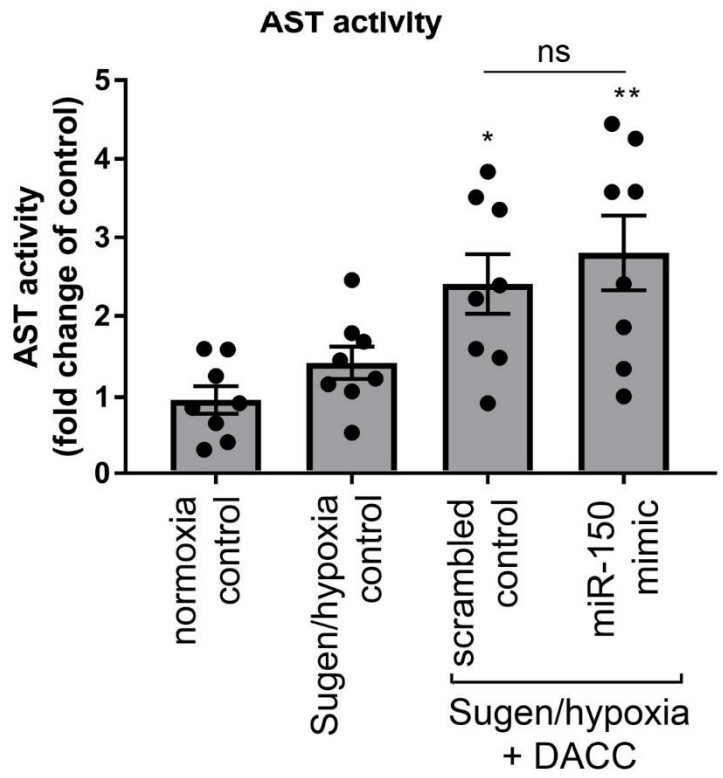

Figure S3. Liver aspartate aminotransferase (AST) activity in mice. AST activity (nmole glutamate/min/mL) was measured in liver tissues from untreated normoxic mice, untreated Sugen/hypoxia mice, Sugen/hypoxia mice treated with DACC/miRNA scrambled controls and Sugen/hypoxia mice treated with DACC/miR-150 mimic (N=8/group). AST activity is shown as fold-change of normoxic controls. Bars are means ± SEM. *$p<0.05$; **$p<0.01$, comparison with normoxic control; one-way ANOVA with Tukey post-test.

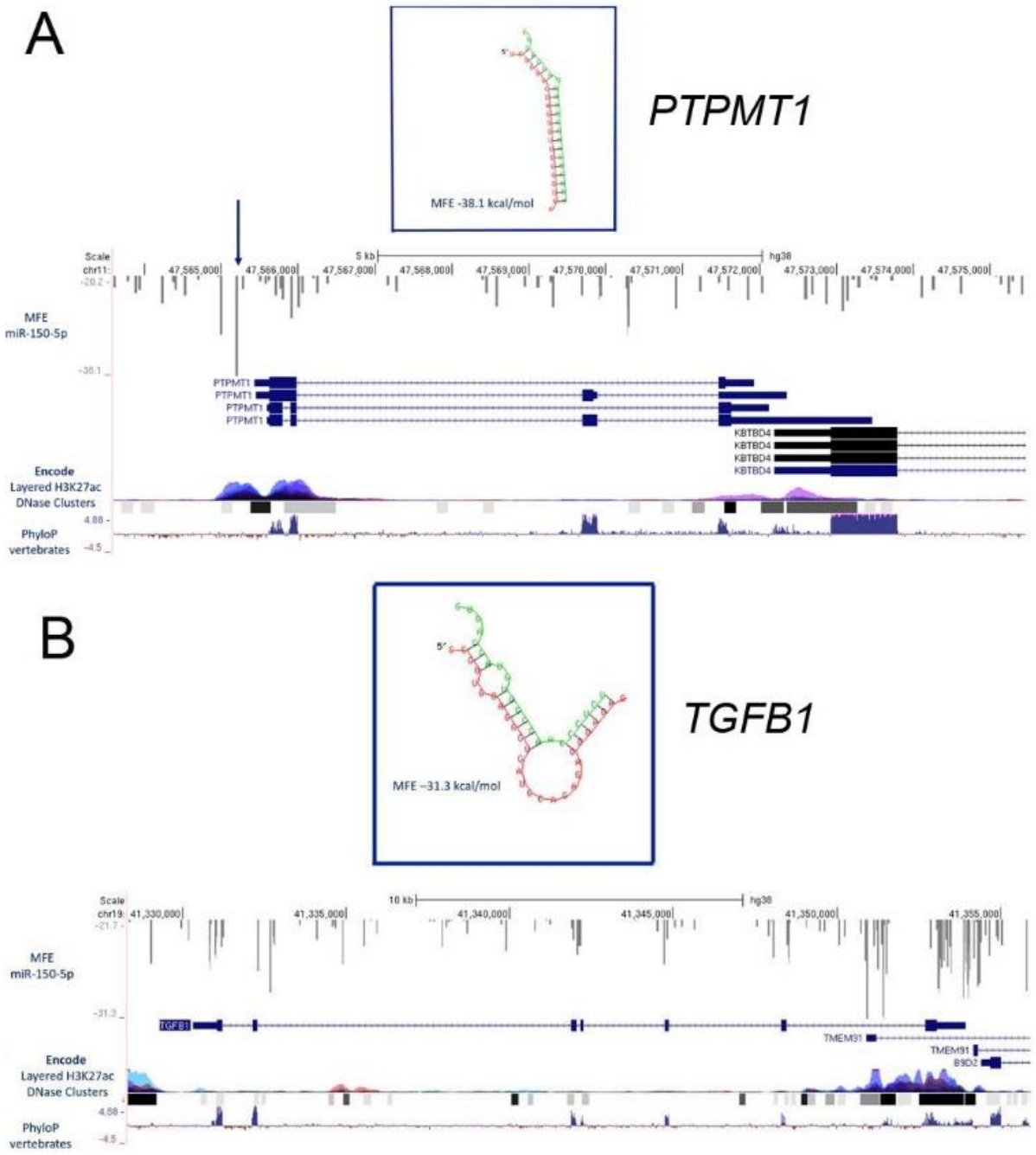

Figure S4. Predicted miR-150 binding sites.

(A) RNAhybrid identified 70 putative miR-150 (MIMAT0000451) predicted binding sites to *PTPMT1* (chr11:47563600-47575461) with minimum free energy (MFE) ranges between -20.2 kcal/mole and -38.1 kcal/mole. The top MFE event (-31.3 kcal/mole) occurred within the promoter region of *PTPMT1* (inset). (B) RNAhybrid gave in-silico predictions for 100 binding events of miR-150 to TGFB1 (chr19:41328324-41355922) with MFE ranges between -21.7 kcal/mole and -31.3 kcal/mole. The top MFE event (-31.3 kcal/mole) occurred within the first intron of TGFB1 (inset). Images are adapted from UCSC Genome Browser.

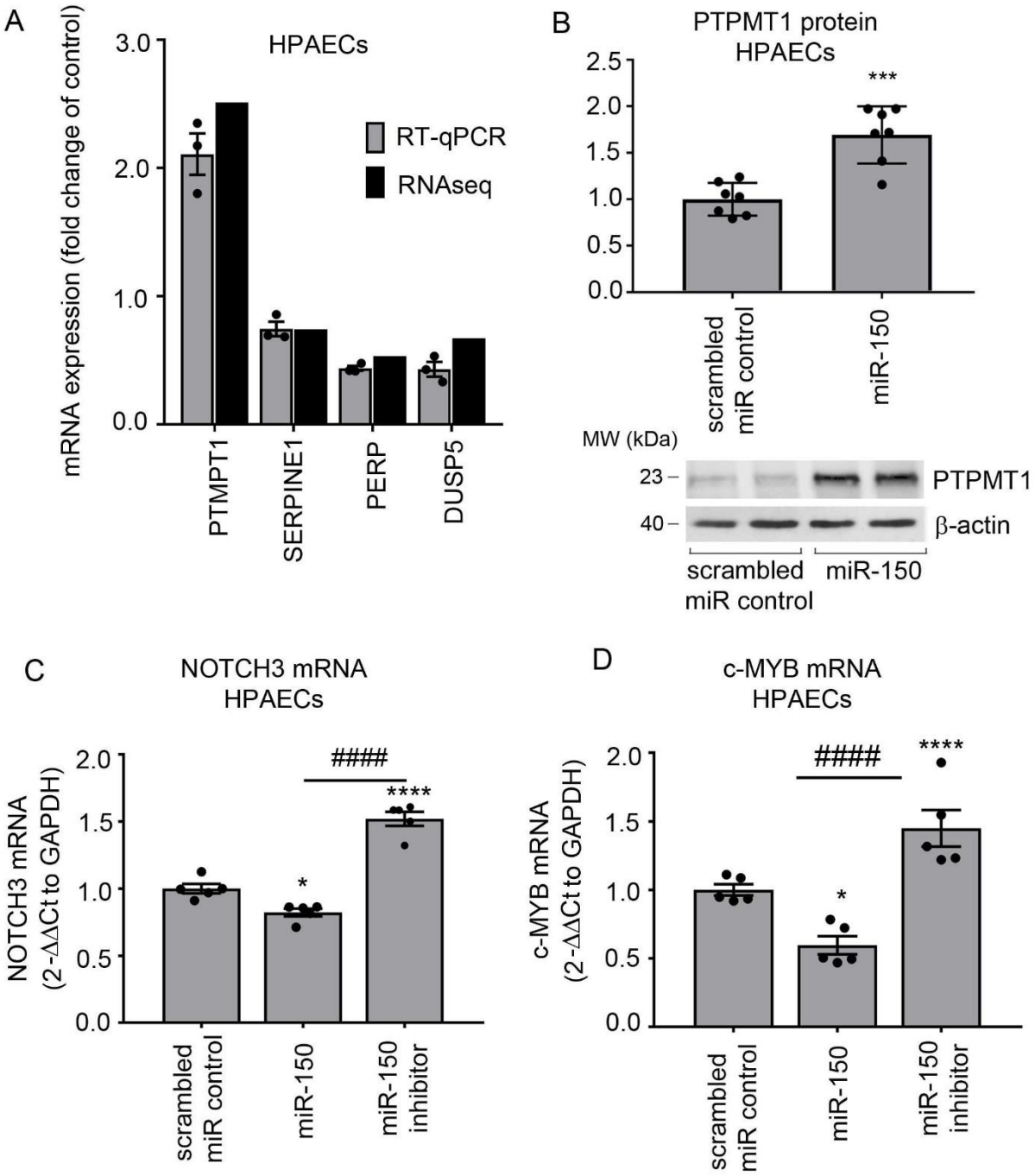

Figure S5. RNA-Sequencing validation. RNA-Sequencing results (black bars) were validated for selected miR-150-regulated genes by RT-qPCR. (A) PTPMT1, SERPINE1, PERP, DUSP5 mRNA levels expressed as fold change of transfection control. (B) Graph shows fold-change in PTPMT1 protein expression in HPAECs transfected with miR-150 mimic, compared with scrambled control. A corresponding representative western blot is shown below the graph. (C) NOTCH3 and (D) c-MYB in HPAECs transfected with scrambled miR, miR-150 mimic or inhibitor. Bars are mean fold-changes of control ± SEM. *p<0.05; ****p<0.0001, comparison with scrambled control; $^{\#\#\#\#}$p<0.0001, comparison, as indicated. In (A) N=3, in (B) N=7, in (C, D) N=5.

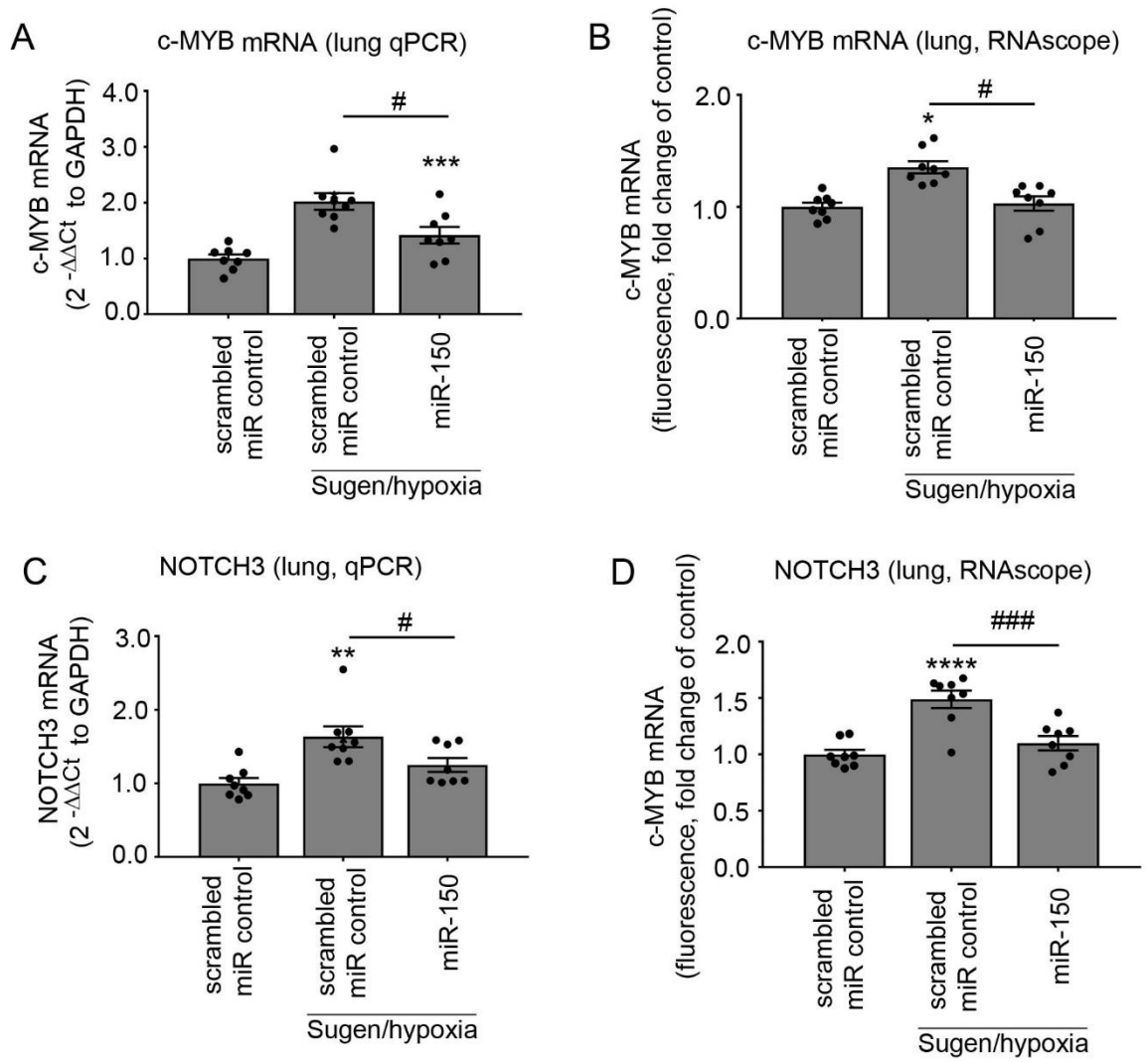

Figure S6. Expression levels of miR-150-regulated genes *in vivo*. (A, B) c-MYB and (C, D) NOTCH3 mRNA evaluated by qPCR or RNAscope in situ hybridization analysis in lungs of healthy controls and Sugen/hypoxia mice treated, as indicated. Bars are mean fold-changes of control ± SEM. *$p<0.05$; ****$p<0.0001$, comparison with scrambled control; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, comparisons, as indicated. N=8.

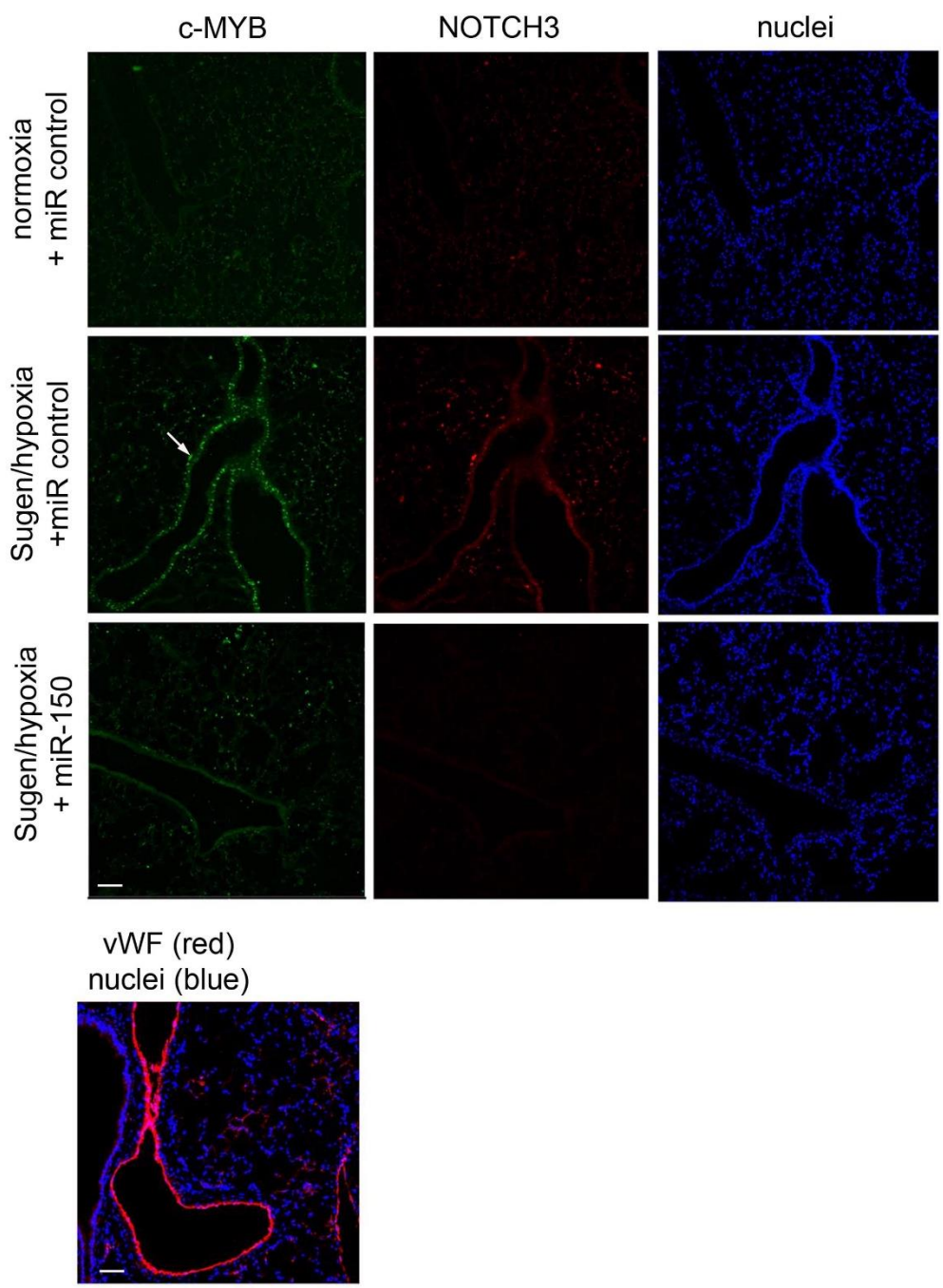

Figure S7. Representative images of c-MYB (green) and NOTCH3 (red) mRNA staining in mouse lungs. Transcripts were identified by the RNAscope fluorescent *in situ* hybridization in lungs from normoxic and Sugen/hypoxia control mice (treated with DACC/scrambled miRNA control) and Sugen/hypoxia mice treated with DACC/miR-150, as indicated. Nuclei are stained in blue (DAPI) N=6. Image bellow the main panel shows vWF staining (red) in healthy lung to visualise distribution of endothelial cells. Bar=50µm.

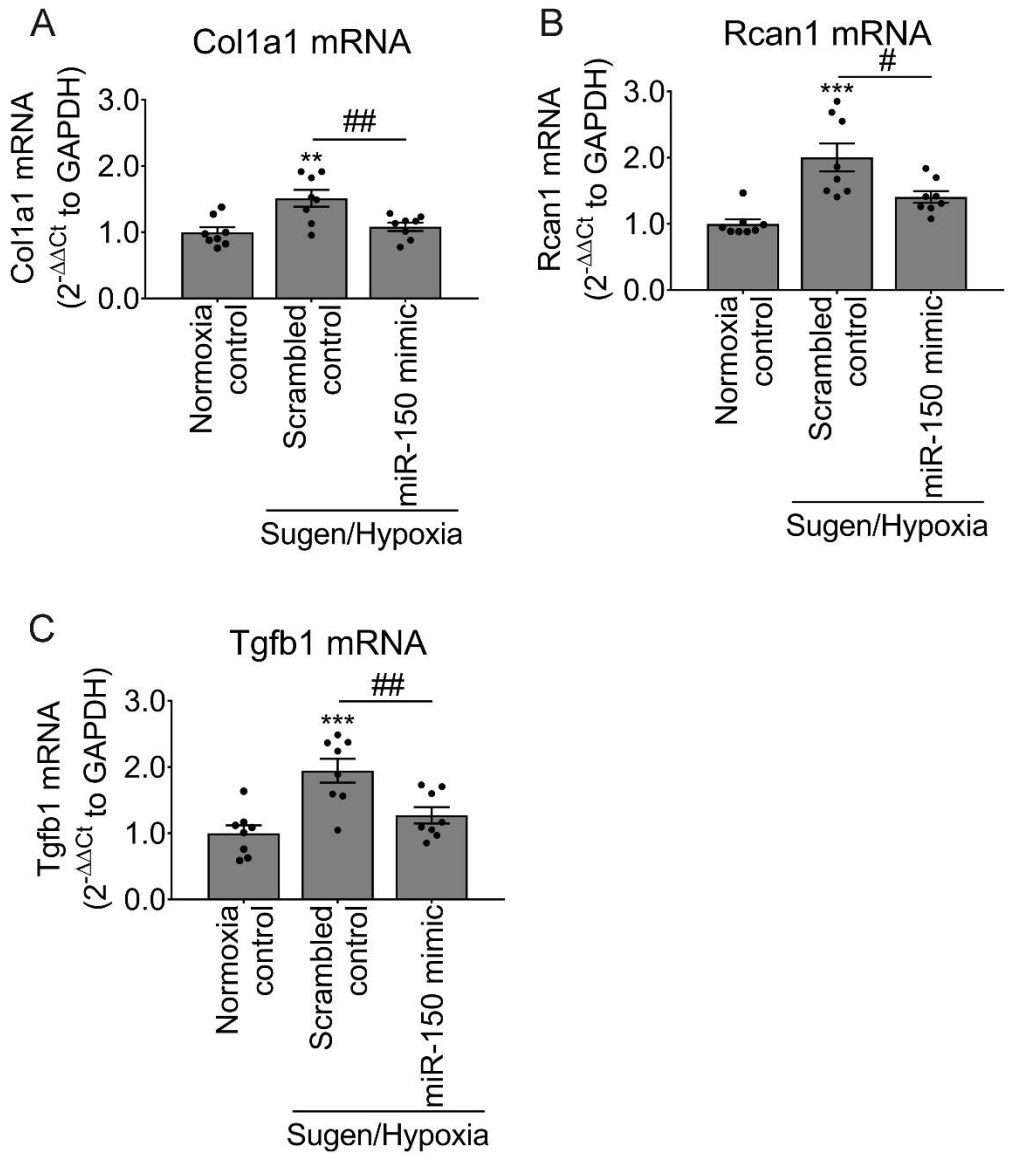

Figure S8. miR-150 supplementation reduces expression of pro-fibrotic genes in the right ventricle of Sugen/hypoxia mice. (A-C) show expression changes in Collagen 1 (Col1a1), regulator of calcineurin 1 (Rcan1) and TGFB1. In graphs, bars are mean fold-changes of control ± SEM; one-way ANOVA with Tukey post-test. ***$p<0.001$, comparison with normoxia control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, comparisons with scrambled control. N=8

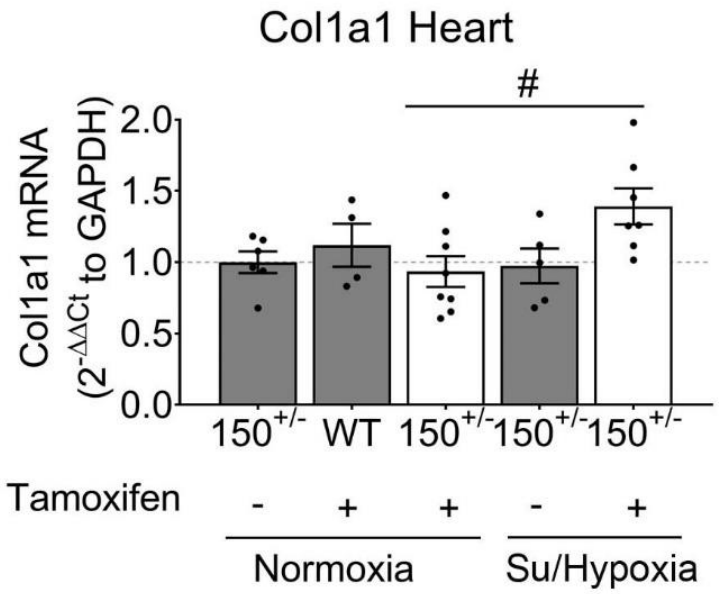

Figure S9. Endothelial deletion of miR-150 increases collagen 1 expression in the heart. Col1a1 mRNA levels in the right ventricle of wildtype and miR-150iEC-KO (miR-150$^{+/-}$) mice in normoxia or Sugen/hypoxia, with and without tamoxifen, as indicated. Empty bars mark miR-150-deficient animals. Bars show mean fold-changes of control ± SEM; one-way ANOVA with Tukey post-test. $^{\#}p<0.05$, comparisons, as indicated. N=5-8.

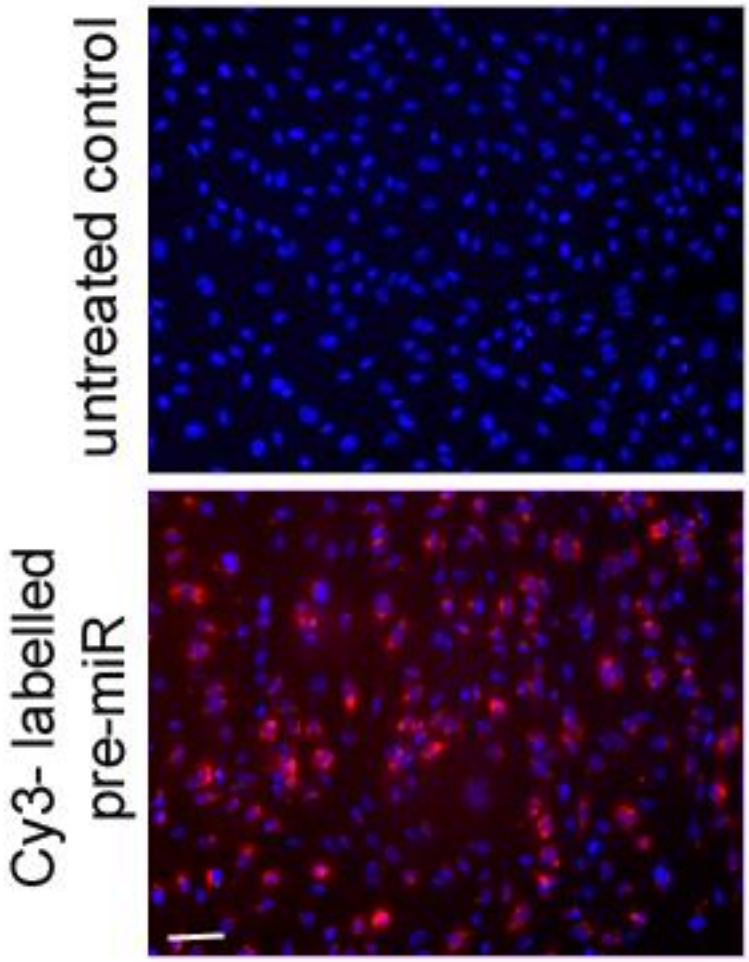

Figure S10. Transfection efficiency in HPAECs transfected with fluorescently-labelled Cy3-pre-miR mimic (20 nM; lower panel). Transfected cells are red, whereas nuclei are blue (DAPI). Bar=50 μm.

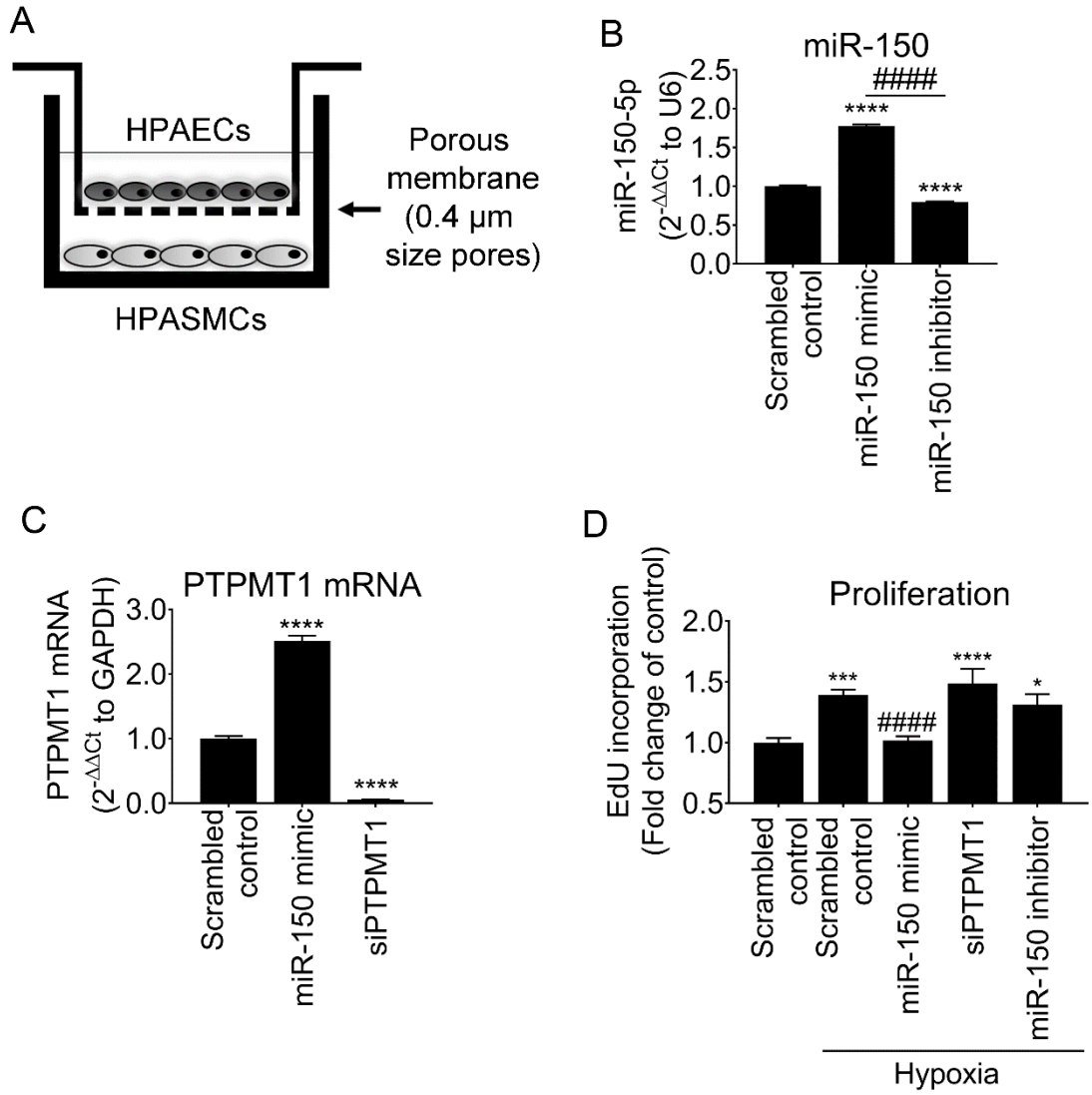

Figure S11. Effects of miR-150 transfection and PTPMT1 silencing on smooth muscle cell proliferation. (A) Schematic representation of non-contact co-culture of HPAECs and HPASMCs. Endothelial cells were seeded in the top chamber, whereas smooth muscle cells were seeded in the bottom of the plate. A porous (0.4 μm pore size) membrane separated the two cell types. (B) miR-150 expression levels in HPASMCs transfected with miR-150 mimic or inhibitor. (C) PTPMT1 expression levels in smooth muscle cells transfected with miR-150 mimic or siPTPMT1. (D) Proliferation of HPASMCs co-cultured with HPAECs transfected with miR-150 mimic, siPTPMT1 and miR-150 inhibitor for 48h. One-way ANOVA with Tukey post-test. Bars are means ± SEM. N =3 in (B and C) and N=5 in (D).

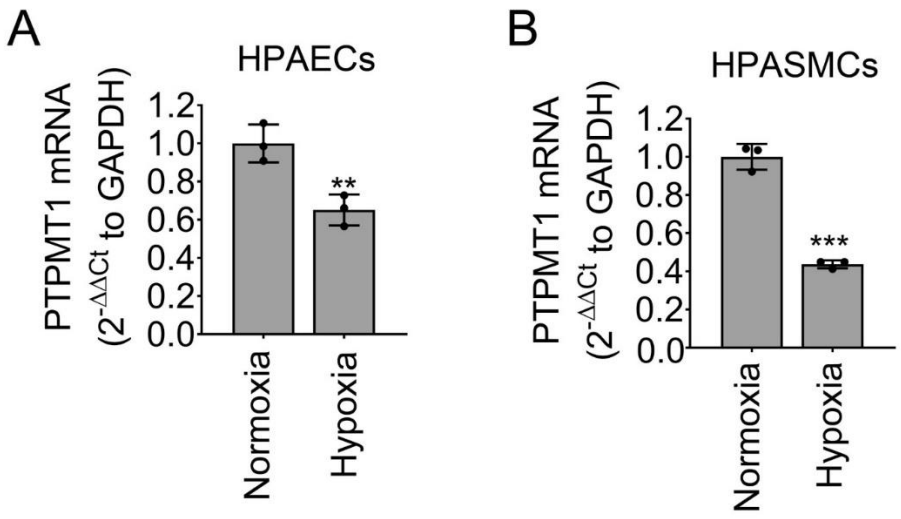

Figure S12. Effects of hypoxia on PTPMT1 expression in HPAECs and HPASMCs. PTPMT1 mRNA expression was measured in (A) HPAECs and (B) HPASMCs under normoxic or hypoxic (24 hours) conditions. In graphs, bars are mean fold-changes of control ± SEM; Student t-test. **p<0.01; ***p<0.001, comparison with normoxic controls, N=3.

Supplementary References

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