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Extended Data Figures and Supplementary Tables

Branched chain α-ketoacids aerobically activate HIF1α signaling in vascular cells

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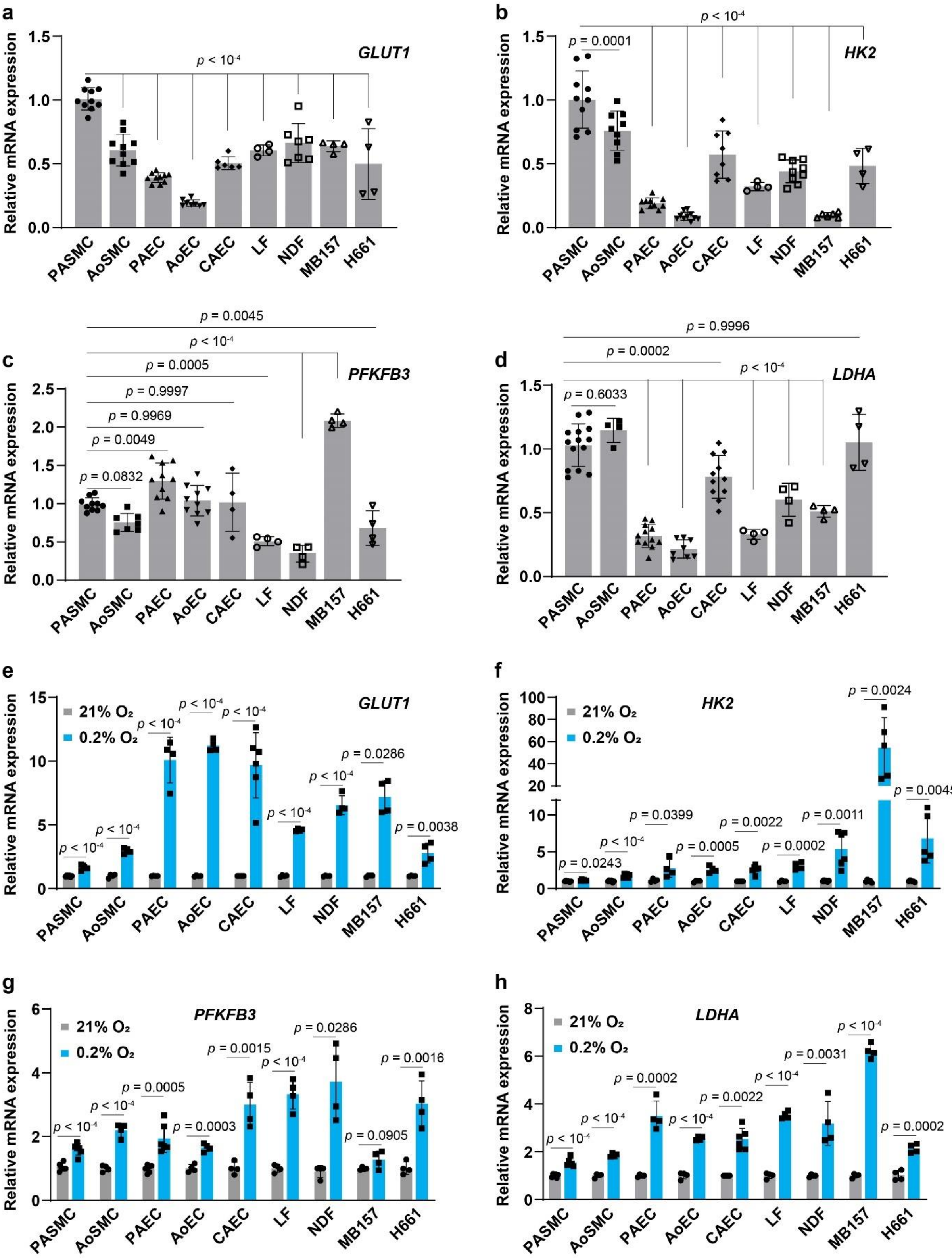
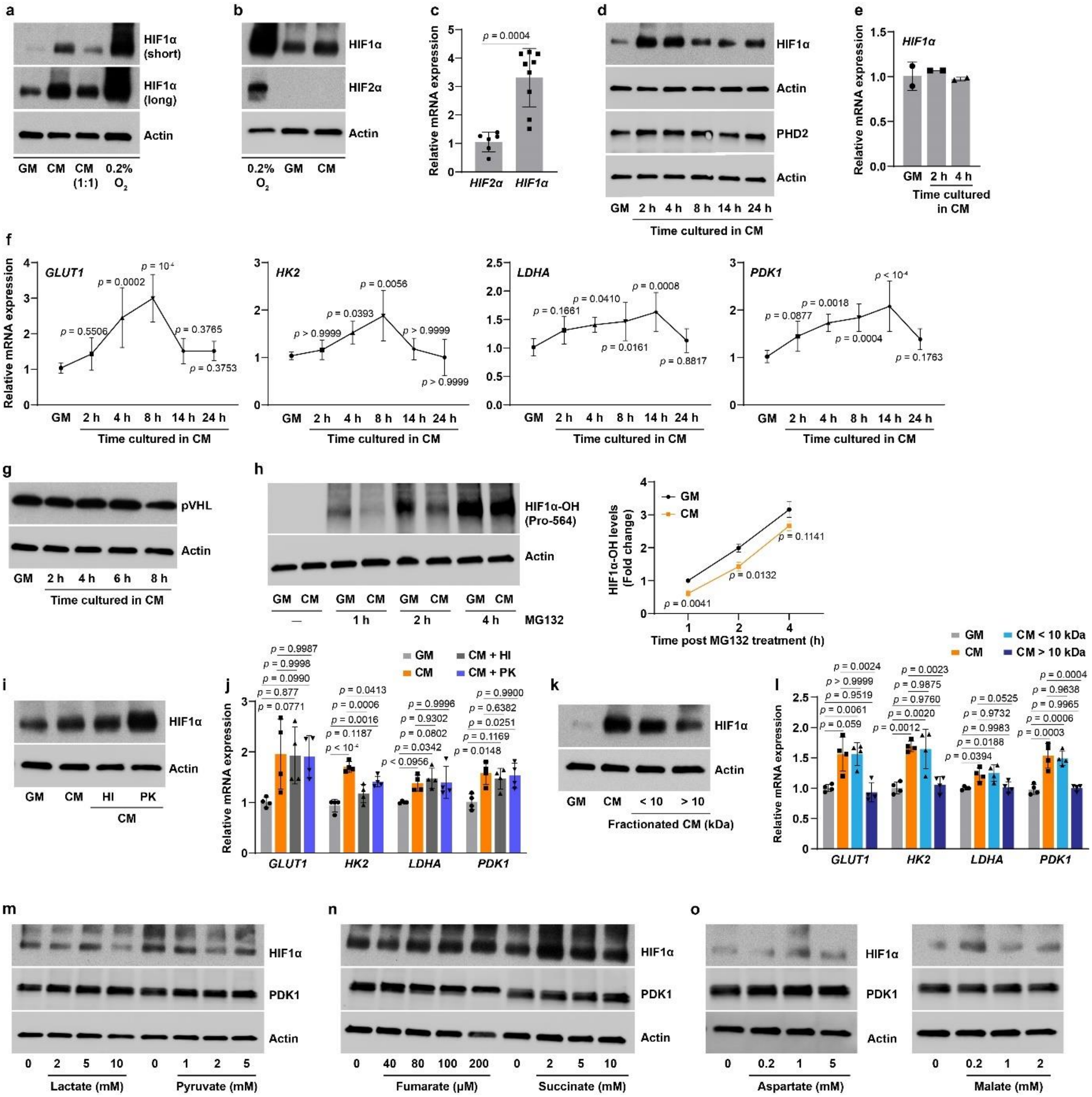

a
GLUT1
Relative mRNA expression
$p < 10^{-4}$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
b
HK2
Relative mRNA expression
$p < 10^{-4}$
$p = 0.0001$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
c
PFKFB3
Relative mRNA expression
$p = 0.0045$
$p < 10^{-4}$
$p = 0.0005$
$p = 0.9997$
$p = 0.9969$
$p = 0.0049$
$p = 0.0832$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
d
LDHA
Relative mRNA expression
$p = 0.9996$
$p = 0.0002$
$p < 10^{-4}$
$p = 0.6033$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
e
GLUT1
21% O_2
0.2% O_2
Relative mRNA expression
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p = 0.0286$
$p = 0.0038$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
f
HK2
21% O_2
0.2% O_2
Relative mRNA expression
$p = 0.0024$
$p = 0.0045$
$p = 0.0399$
$p = 0.0022$
$p = 0.0011$
$p < 10^{-4}$
$p = 0.0005$
$p = 0.0002$
$p = 0.0243$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
g
PFKFB3
21% O_2
0.2% O_2
Relative mRNA expression
$p = 0.0286$
$p = 0.0015$
$p < 10^{-4}$
$p = 0.0016$
$p = 0.0005$
$p < 10^{-4}$
$p = 0.0905$
$p < 10^{-4}$
$p = 0.0003$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661
h
LDHA
21% O_2
0.2% O_2
Relative mRNA expression
$p < 10^{-4}$
$p = 0.0031$
$p = 0.0002$
$p < 10^{-4}$
$p = 0.0022$
$p < 10^{-4}$
$p < 10^{-4}$
$p < 10^{-4}$
$p = 0.0002$
PASMC AoSMC PAEC AoEC CAEC LF NDF MB157 H661

Extended Data Fig.1 VSMCs exhibit high abundance of key glycolysis regulatory genes and are resistant to hypoxia-induced upregulation of these genes.

a-d, Basal mRNA expression of glycolytic genes *GLUT1* (**a**), *HK2* (**b**), *PFKFB3* (**c**), and *LDHA* (**d**) in 9 different cell types under aerobic conditions. Fold change was calculated relative to PASMCs. *n* = 4-14.

e-h, mRNA expression of glycolytic genes in 9 different cell types cultured under normoxia (21% O_2) or hypoxia (0.2% O_2). Fold change was calculated relative to the corresponding type of cells grown under aerobic condition. *n* = 4-6.

One-way ANOVA followed by Dunnett's post-hoc test (**a-d**), Student's t test or Mann-Whitney U test (**e-h**) was applied when compared to untreated PASMCs (**a-d**) or normoxic cultures of the matched cell type (**e-h**).



Extended Data Fig. 2 Medium conditioned from PASMCs induces aerobic activation of HIF1α signaling.

a, HIF1α protein expression in PASMCs cultured in growth medium (GM), conditioned medium (CM), or 1:1 (v/v) mix of CM and GM (CM 1:1). Cells grown under 0.2% O_2 were used as positive controls. Short (20 min) and long (60 min) represent film exposure duration.

b, HIF1α and HIF2α protein levels in cells cultured in GM, CM, or 0.2% O_2.

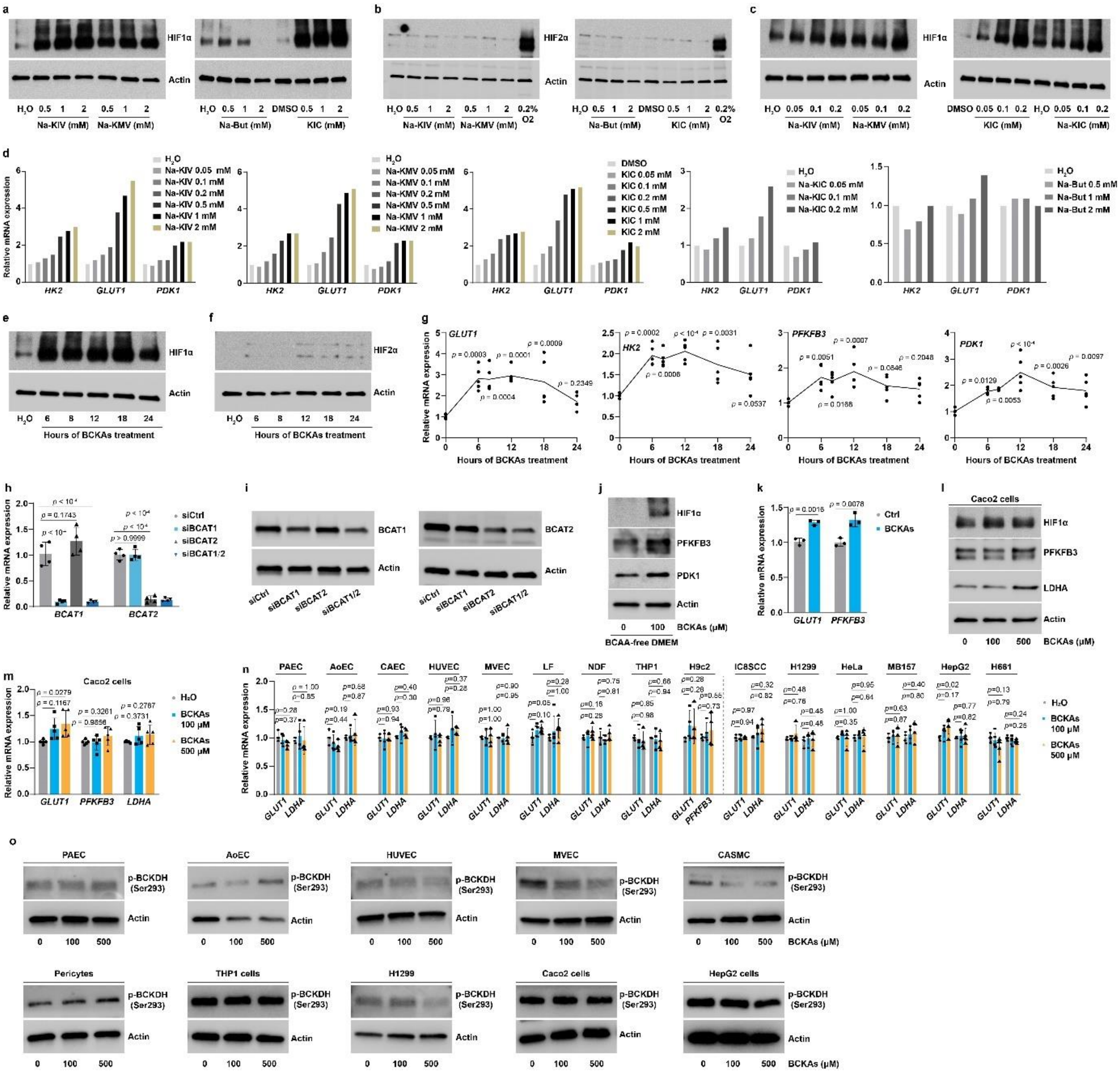
c, *HIF1α* and *HIF2α* mRNA abundance in PASMCs cultured in GM. Fold change was calculated relative to HIF2α. n = 6-9.

d, HIF1α and PHD2 protein levels in PASMCs cultured in CM for 2-24 hours.

e, *HIF1α* mRNA expression in cells cultured in CM for 2 and 4 hours. Fold change was calculated relative to PASMCs grown in GM. n = 2.

f, mRNA expression of HIF1α target genes in glucose metabolism in PASMCs when cultured in CM for various time points. Fold change was calculated relative to cells in GM. $n = 6$.
g, von-Hippel Lindau protein (pVHL) levels in cells cultured in CM for different times.
h, Representative immunoblots and quantitation of hydroxylated HIF1α protein (HIF1α-OH Pro-564) levels in GM- or CM-cultured PASMCs after addition of proteasomal inhibitor MG132 (20 μM) for 1-4 hours. Fold change was calculated relative to GM-cultured PASMCs at 1 hour of MG132 incubation. $n = 3$.
i,j HIF1α protein levels (**i**) and mRNA expression of its transcriptional targets (**j**) in GM, CM, and heat inactivation (HI) or proteinase K (PK) treated CM cultured PASMCs. Fold change in **j** was calculated relative to GM-cultured cells. $n = 4$ (**j**).
k,l HIF1α protein levels (**k**) and mRNA expression of its transcriptional targets (**l**) in GM, CM, and fractionated CM (larger than 10 kDa fraction, >10 and less than 10 kDa fraction, < 10) cultured PASMCs. Fold change in **l** was calculated relative to GM-cultured cells. $n = 4$ (**l**).
m-o, HIF1α and its target PDK1 protein levels in PASMCs treated with lactate or pyruvate (**m**), fumarate or succinate (**n**), aspartate or malate (**o**) as indicated doses.

Mann-Whitney U test (**c**), Student t test (**h**), or one-way ANOVA followed by Tukey's post-hoc test or Kruskal-Wallis test followed by Dunn's test (**f**, **j**, **l**) was applied when compared to HIF2α in PASMCs (**c**), to GM-cultured PASMCs with time-matched MG132 treatment (**h**) or no treatment (**f**, **j**, **l**) or to CM-cultured PASMCs (**j**, **l**).



Extended Data Fig.3 BCKAs are the mediators of paracrine activation of HIF1α signaling under aerobic conditions.

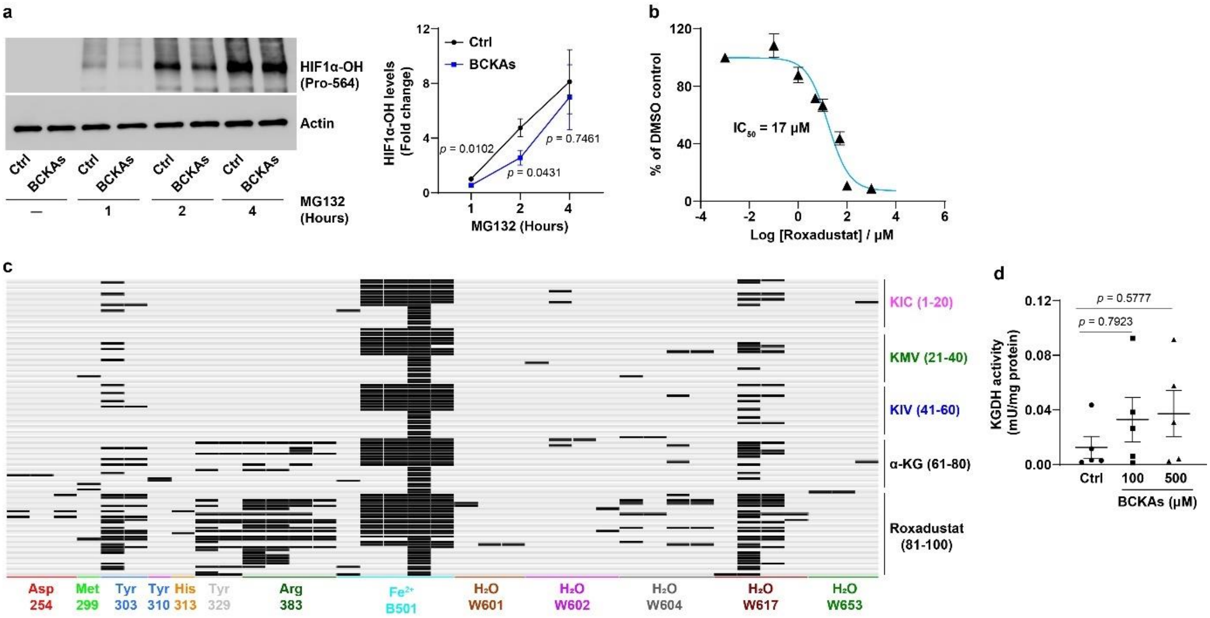
a-c, HIF1α (**a, c**) and HIF2α (**b**) proteins in PASMCs treated with 0.05-2 mM of sodium salts of KIV (Na-KIV), KMV (Na-KMV), butyrate (Na-But), KIC (Na-KIC), or acid form of KIC (KIC; DMSO as vehicle control) for 8 hours. Hypoxia (0.2% O_2) induced HIF2α protein stabilization was included for comparison.

d, mRNA expression of three HIF1α target genes in glucose metabolism in PASMCs stimulated with 0.05-2 mM of Na-KIV, Na-KMV, Na-KIC, KIC, and Na-But for 8 hours. Fold change was calculated relative to vehicle control (H_2O or DMSO) treated cells. *n* = 1.

e-g, HIF1α (**e**) and HIF2α (**f**) protein levels and the mRNA expression of HIF1α target genes in glucose metabolism (**g**) in PASMCs stimulated with BCKAs (100 μM Na-KIC, 50 μM of each Na-KIV and Na-KMV) for different time points. Fold change in **g** was calculated relative to untreated control cells at 8-hour time point. *n* = 5 (**g**).

h,i, mRNA (**h**) and protein (**i**) expression of BCAT1 and BCAT2 in PASMCs transfected with siRNAs for control (siCtrl), *BCAT1* (siBCAT1), *BCAT2* (siBCAT2), or both (siBCAT1/2). Fold change in **h** was relative to siCtrl-transfected cells. *n* = 4 (**h**).
j,k, Protein levels of HIF1α, PFKFB3, and PDK1 (**j**) and mRNA expression of *GLUT1* and *PFKFB3* (**k**) in PASMCs cultured in BCAA-free DMEM in the presence or absence of BCKAs for 8 hours. Fold change in **k** was calculated relative to untreated control cells. *n* = 3 (**k**).
l,m, HIF1α, PFKFB3, and LDHA protein levels (**l**) and the mRNA expression of HIF1α regulatory genes in glycolysis (**m**) of human colorectal adenocarcinoma Caco2 cells treated with BCKAs. Fold change in (**m**) was relative to vehicle control treated cells. *n* = 5 (**m**).
n, mRNA expression of *GLUT1*, *LDHA*, or *PFKFB3* in normal and cancerous cells after stimulation with different doses of BCKAs. Fold change was calculated relative to their own untreated control cells. Dotted line separates normal *vs.* malignant cell types. *n* = 4-5.
o, Phosphorylated BCKDH (p-BCKDH) protein levels in 10 different types of cells with BCKA treatment.

One-way ANOVA followed by Dunnett's post-hoc test (**g**, **h**, **m**, **n**) or Student's t test (**k**) was applied when compared to untreated control PASMCs (**g**), siCtrl-transfected PASMCs (**h**), BCAA-free DMEM-cultured control PASMCs (**k**), untreated Caco2 cells (**m**), or the corresponding untreated cells (**n**).



Extended Data Fig. 4 Effects of BCKAs on PHD2 and KGDH activity.

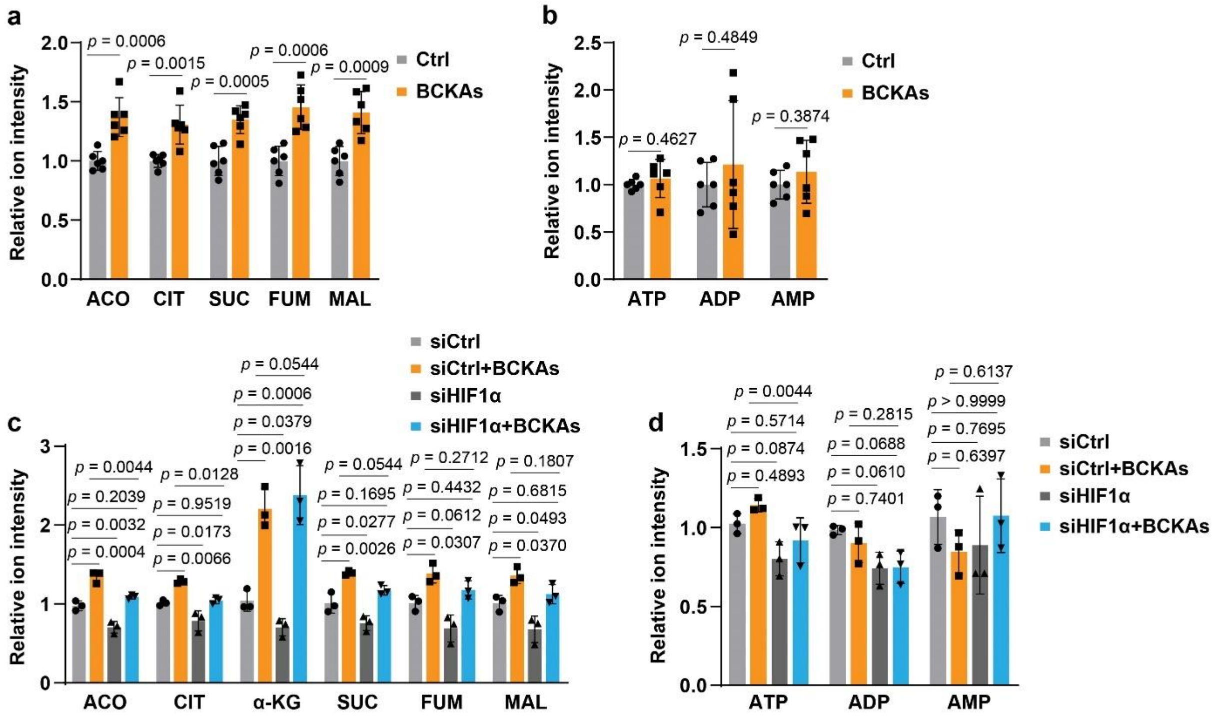
a, PASMCs were treated with BCKAs (100 μM of KIC, 50 μM of each KIV and KMV) or vehicle control for 8 hours followed by addition of proteasomal inhibitor MG132 (20 μM) for 1-4 hours. Hydroxylated HIF1α (HIF1α-OH Pro-564) protein levels were measured and quantitated. Fold change was calculated relative to untreated cells with MG132 incubation for 1 hour. *n* = 3.

b, Inhibition curve and IC_{50} value of roxadustat for PHD2 hydroxylase activity. *n* = 4.

c, Protein-ligand interaction fingerprint (PLIF) prediction of 20 potential binding configurations of each BCKA with the PHD2 active site. α-KG and roxadustat, two known ligands of PHD2 enzyme, were included for comparison.

d, KGDH activity in PASMCs treated with BCKAs. *n* = 5.

Student's t test (**a**) or Kruskal-Wallis test followed by Dunn's post-hoc test (**d**) was applied when compared to untreated PASMCs at time-matched MG132 treatment (**a**) or untreated PASMCs (**d**).

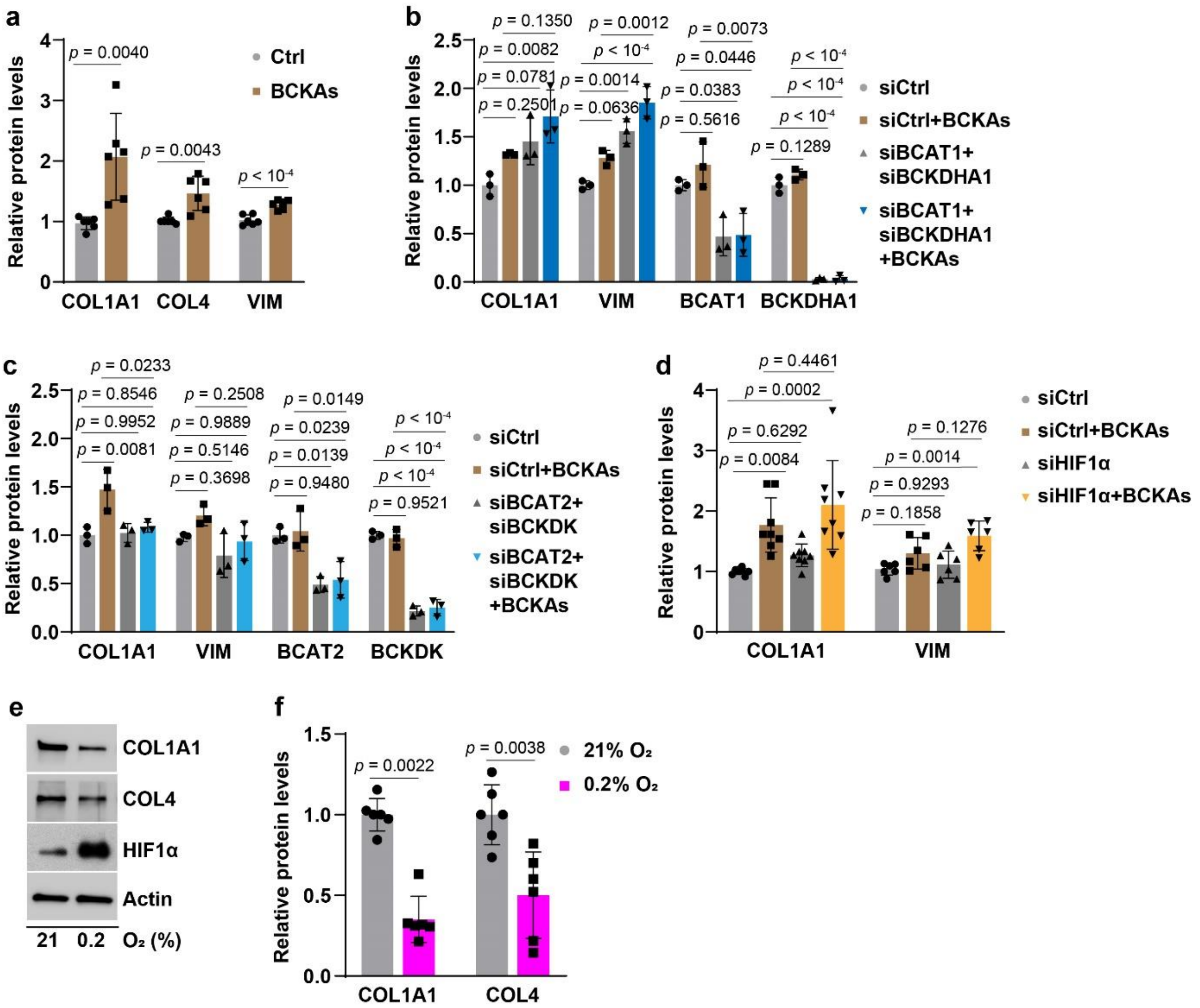


Extended Data Fig. 5 The influence of BCKAs on mitochondrial respiration and its dependence on HIF1α activity in PASMCs.

a,b, LC-MS measurements of intermediary metabolites aconitate (ACO), citrate (CIT), succinate (SUC), fumarate (FUM), and malate (MAL) of the TCA cycle (**a**), and of ATP and its derivatives (**b**) in PASMCs in the presence or absence of BCKAs. Fold change was calculated relative to control cells. $n = 6$.

c,d, PASMCs were transfected with human *HIF1α* siRNA (siHIF1α) or control siRNA (siCtrl) followed by treatment with BCKAs. LC-MS was used to measure the TCA cycle metabolites (**c**), and ATP and its metabolites (**d**). Fold change was calculated relative to siCtrl-transfected and untreated cells. $n = 3$.

Student's t test (**a**, **b**) or one-way ANOVA followed by Tukey's post-hoc test (**c**, **d**) was applied when compared to control PASMCs (**a**, **b**), or siCtrl-transfected and control or BCKA-treated cells (**c**, **d**).



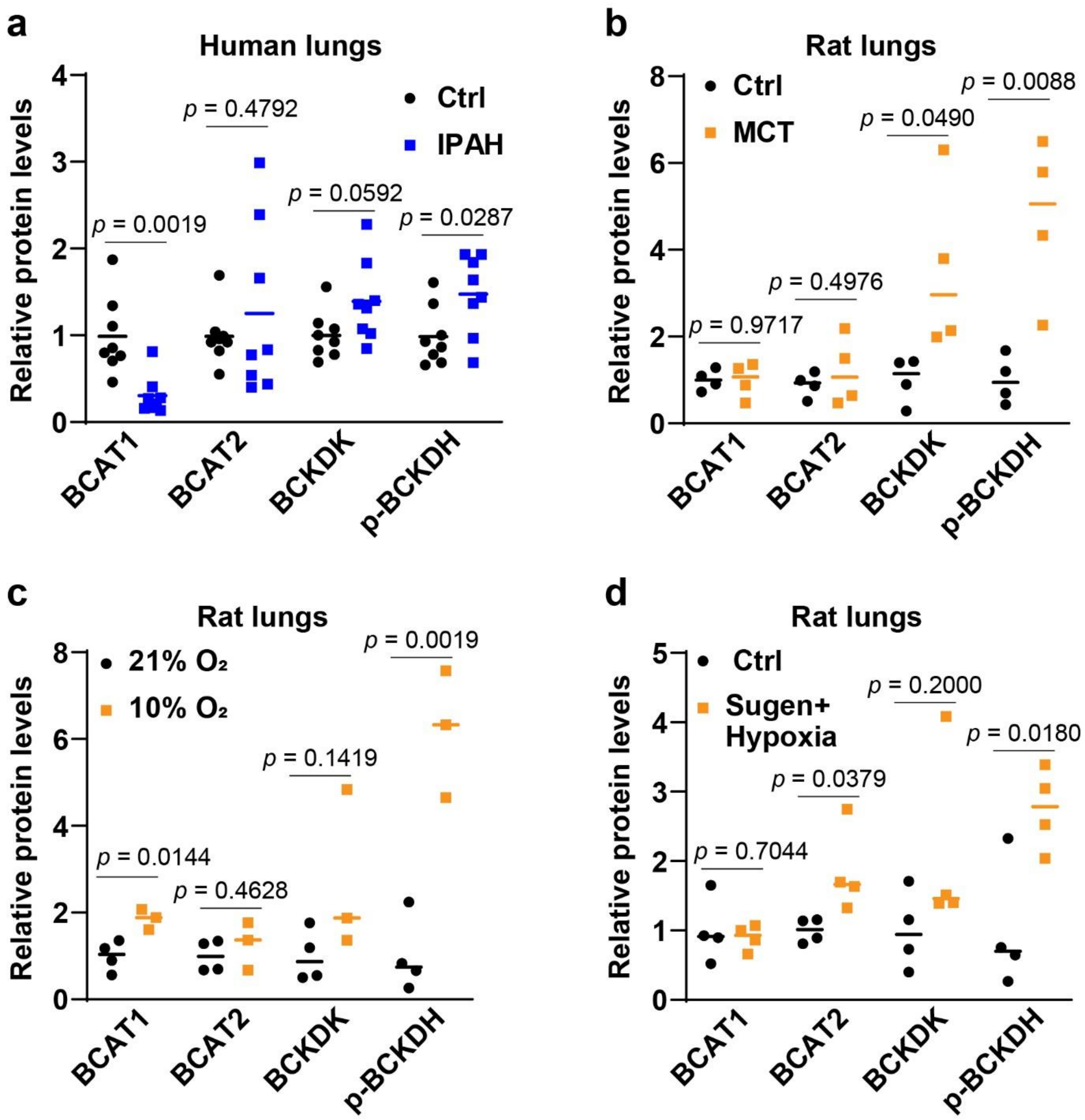
Extended Data Fig. 6 The levels of synthetic phenotype marker proteins in PASMCs.

(a) Protein levels in PASMCs treated with BCKAs. Fold change was calculated relative to untreated control. *n* = 6.

(b-d) Protein levels in PASMCs transfected with control siRNA (siCtrl), *BCAT1* and *BCKDHA1* siRNA (siBCAT1+siBCKDHA1; **b**), or *BCAT2* and *BCKDK* siRNA (siBCAT2+siBCKDK; **c**), or *HIF1α* siRNA (siHIF1α; d) with or without BCKA treatment. Fold change was calculated relative to siCtrl-transfected and untreated control. *n* = 3 (**b, c**) and 8 (**d**).

(**e,f**) Representative immunoblots (**e**) and quantitation (**f**) of COL1A1 and COL4 protein levels in PASMCs cultured in 21% O_2 or 0.2% O_2. HIF1α protein was included as a positive control in hypoxia. Fold change in **f** was relative to normoxic cultures of PASMCs. *n* = 6.

Student's t test (COL1A1 and VIM in **a**, COL4 in **f**), Mann-Whitney U test (COL4 in **a**, COL1A1 in **f**), or one-way ANOVA followed by Tukey's post-hoc test (**b-d**) was applied when compared to control PASMCs (**a**, **f**), or siCtrl-transfected and control or BCKA-treated cells (**b-d**).

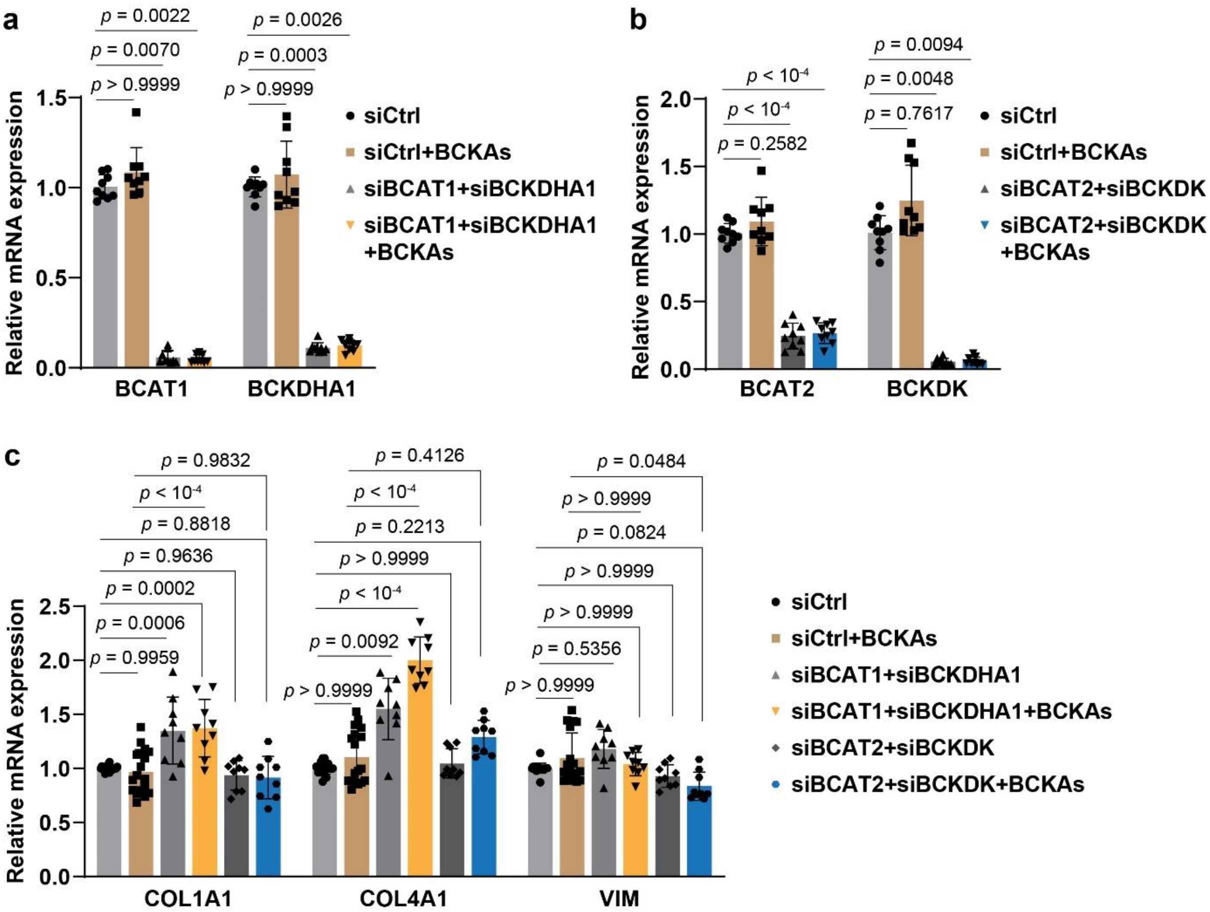


Extended Data Fig. 7 The levels of key BCKA metabolic proteins in the lungs of PAH patients and rats.

a, Quantitation results of BCAT1, BCAT2, BCKDK, and p-BCKDH proteins in the lungs of IPAH patients. *n* = 8 individuals.

b-d, Quantitation results of BCAT1, BCAT2, BCKDK, and p-BCKDH proteins in the lungs of PAH rats treated with MCT (**b**), hypoxia (10% O_2; **c**), or Sugen5416+hypoxia (**d**). *n* = 3-4 rats.

Student's t test (**a-d**) or Mann-Whitney U test (BCAT1 in **a** and BCKDK in **d**) was applied when compared to control patients (**a**) or animals (**c-d**).



Extended Data Fig. 8 The expression of synthetic marker genes in IPAH-PASMCs with endogenous and exogenous manipulation of BCKA levels.

(**a**) mRNA expression of BCAT1 and BCKDHA1 in IPAH-PASMCs transfected with siRNAs for control (siCtrl) or *BCAT1* and *BCKDHA1* (siBCAT1+siBCKDHA1) followed by BCKAs or vehicle control treatment. $n = 9$ from 3 individuals.

(**b**) mRNA expression of BCAT2 and BCKDK in IPAH-PASMCs transfected with siCtrl or *BCAT2* and *BCKDK* (siBCAT2+siBCKDK) followed by BCKAs or vehicle control treatment. $n = 9$ from 3 individuals.

(**c**) mRNA expression of synthetic marker genes in IPAH-PASMCs transfected and treated as described in panels **a** and **b**. n = 9-18 from 3 individuals.

Kruskal-Wallis test followed by Dunn's post-hoc test (**a-c**) or one-way ANOVA followed by Dunnett's (**b**) or Tukey's (**c**) post-hoc test was applied when compared to siCtrl-transfected control cells (**a-c**) or BCKA-treated cells (**c**).

Supplementary Table 1 Clinical and demographic information on human specimen presented in this study

Patient ID	Clinical diagnosis	Gender	Race	Ethnicity
Lung RNA samples				
AH-007	FDL	M	White	Non-Hispanic
AH-009	FDL	M	White	Non-Hispanic
AH-012	FDL	M	White	Non-Hispanic
AH-013	FDL	F	White	Non-Hispanic
BA-033	FDL	M	White	Non-Hispanic
BA-040	FDL	M	Unknown	Hispanic or Latino
BA-046	FDL	F	Unknown	Hispanic or Latino
BA-055	FDL	M	White	Non-Hispanic
UC-010	FDL	F	White	Non-Hispanic
VA-005	FDL	M	White	Non-Hispanic
BA-017	IPAH	F	White	Non-Hispanic
CC-017	IPAH	M	White	Non-Hispanic
CC-030	IPAH	F	White	Non-Hispanic
ST-004	IPAH	F	White	Non-Hispanic
ST-010	IPAH	M	White	Non-Hispanic
ST-017	IPAH	M	White	Non-Hispanic
ST-019	IPAH	M	White	Hispanic or Latino
ST-042	IPAH	M	White	Non-Hispanic
UA-013	IPAH	M	Asian	Non-Hispanic
VA-015	IPAH	F	White	Non-Hispanic
Frozen lung tissues				
AH-012	FDL	M	White	Non-Hispanic
AH-013	FDL	F	White	Non-Hispanic
AH-016	FDL	M	White	Non-Hispanic
BA-040	FDL	M	Unknown	Hispanic or Latino
BA-043	FDL	M	Unknown	Hispanic or Latino
BA-046	FDL	F	Unknown	Hispanic or Latino
BA-048	FDL	M	White	Non-Hispanic
BA-049	FDL	M	Unknown	Non-Hispanic

BA-055	FDL	M	White	Non-Hispanic
BA-062	FDL	M	Asian	Unknown
BA-017	IPAH	F	White	Non-Hispanic
CC-030	IPAH	F	White	Non-Hispanic
ST-028	IPAH	F	White	Hispanic or Latino
ST-033	IPAH	F	White	Non-Hispanic
ST-037	IPAH	F	Unknown	Hispanic or Latino
ST-042	IPAH	M	White	Non-Hispanic
ST-052	IPAH	M	Asian	Non-Hispanic
UA-013	IPAH	M	Asian	Non-Hispanic
VA-011	IPAH	F	White	Non-Hispanic
VA-015	IPAH	F	White	Non-Hispanic
Lung slides				
BA-049	FDL	M	Unknown	Non-Hispanic
BA-062	FDL	M	Asian	Unknown
BA-046	FDL	F	Unknown	Hispanic or Latino
PASMCs				
Patient ID	**Clinical diagnosis**	**Gender**	**Race**	**Age (Y)**
CC-013	IPAH	F	White	27
ST-019	IPAH	M	White	25
ST-026	IPAH	M	White	40
UA-013	IPAH	M	Asian	18
VA-011	IPAH	F	White	32

FDL: failed donor lung; IPAH: Idiopathic pulmonary arterial hypertension

Supplementary Table 2 Abbreviations and their corresponding full names used

Abbreviation	Full name
α-KG	α-ketoglutarate
ACTA2	α-smooth muscle actin
AoSMCs	aortic smooth muscle cells
BCAAs	branched chain amino acids
BCAT	branched chain amino acid transaminase
BCKAs	branched chain α-ketoacids
BCKDH	branched chain ketoacid dehydrogenase complex
BCKDK	branched chain ketoacid dehydrogenase kinase
CASMCs	coronary artery smooth muscle cells
COL1A1	collagen 1A1
COL4	collagen 4
ECAR	extracellular acidification rate
GLUT1	glucose transporter 1
HK2	hexokinase 2
HIF1α	hypoxia-inducible factor 1α
KGDH	α-KG dehydrogenase
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
KMV	α-keto-β-methylvalerate
L2HG	L-2-hydroxyglutarate
L2HGDH	L2HG dehydrogenase
LDHA	lactate dehydrogenase A
mPAP	mean pulmonary artery pressure
OCR	oxygen consumption rate
PAH	pulmonary arterial hypertension
PASMCs	pulmonary arterial smooth muscle cells
PDK1	pyruvate dehydrogenase kinase 1
PFKFB3	6-phosphofructo-2-kinase/fructose 2,6-biphosphatase 3
PHD2	prolyl hydroxylase domain-containing protein 2
PVR	pulmonary vascular resistance
ROS	reactive oxygen species
TCA	tricarboxylic acid
VIM	vimentin
VSMCs	vascular smooth muscle cells