Aus dem Institut für Phisiologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

The ROCK/PTEN/TRPC6 pathway in hypoxic pulmonary vasoconstriction

zur Erlangung des akademischen Grades

Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 14.09.2018

Table of Contents

Abstract

Aims: Hypoxic pulmonary vasoconstriction (HPV) is a physiological response to hypoxia, which optimizes ventilation-perfusion ratio (V/Q) by the redistribution of blood flow from poorly ventilated to better aerated lung areas. In HPV, increases in intracellular Ca^{2+} concentration in response to hypoxia trigger pulmonary artery smooth muscle cell (PASMC) contraction. Transient receptor potential canonical 6 (TRPC6) cation channels that have translocated to caveolae in the plasma membrane in response to hypoxia have been show to play a critical role in this Ca^{2+} response. Phosphatase and tensin homolog (PTEN) serves as scaffold for TRPC6 and as such, regulates its abundance at the plasma membrane in endothelial cells. Therefore we aimed to define the role of PTEN in HPV, with a specific focus on its role in the recruitment of TRPC6 to caveolae in PASMC.

Methods and results:

Experiments were performed in the isolated perfused mouse lung model (IPL). Smooth muscle specific PTEN deficiency attenuated pulmonary vasoconstriction in response to hypoxia but not to angiotensin II (Ang II). PTEN in human PASMC was knocked down by siRNA and PTEN deficient murine PASMC were isolated. In both cases the increase in intracellular Ca^{2+} concentration in response to hypoxia was attenuated compared to control cells. PTEN-TRPC6 interaction was assessed by co-immunoprecipitation and proximity ligation assay with both assays confirming an increased interaction of PTEN with TRPC6 under hypoxic condition. Caveolae were separated by sucrose gradient centrifugation, and PTEN and TRPC6 were found to translocate to caveolae in response to hypoxia. Rho-kinase (ROCK) inhibition prevented PTEN-TRPC6 interaction, their translocation to caveolae, and the intracellular Ca^{2+} increase in response to hypoxia in PASMC, and attenuated the HPV response in the IPL.

Conclusion: These data implicate a critical role for PTEN in HPV in that hypoxia triggers the interaction of PTEN with TRPC6 in a ROCK dependent manner which is required for the subsequent translocation of TRPC6 to caveolae and the elicitation of Ca^{2+} entry into and hence, contraction of PASMC.

Abstrakt

Ziele: Die hypoxische pulmonale Vasokonstriktion (HPV) ist ein physiologischer Mechanismus, bei dem der Blutfluss von schlecht zu besser belüfteten Lungenarealen umgeleitet wird, um somit ein möglichst optimales Belüftungs-Durchblutungsverhältnis (V/Q) sicher zu stellen.

Dabei transloziert der Kationenkanal transient receptor potential canonical 6 (TRPC6) als Reaktion auf hypoxische Bedingungen zu Caveolen an der Plasmamembran, wo er einen Ca^{2+} Einstrom vermittelt der nachfolgend die Kontraktion der pulmonal-arteriellen glatten Muskelzellen (PASMC) auslöst.

Aufgrund bisheriger Ergebnisse, die eine Regulation von TRPC6 durch die Phosphatase phosphatase and tensin homolog (PTEN) in endothelialen Zellen nachwiesen, soll in der vorliegenden Studie die Rolle von PTEN in PASMC bei der HPV und speziell hinsichtlich der Rekrutierung von TRPC6 in Caveolen untersucht werden.

Methoden und Ergebnisse: Versuche an isoliert-perfundierten Lungen (IPLs) von Mäusen, deren glatte Muskelzellen spezifisch für PTEN defizient waren, zeigten eine verringerte vasokonstriktorische Reaktion auf Hypoxie. Dieser Effekt konnte jedoch nicht nach Stimulation mit Angiotensin II (AngII) nachgewiesen werden. Analog dazu hemmte ein siRNA vermittelter PTEN knockdown in humanen PASMC den Hypoxie-induzierten Anstieg der zytosolischen Ca^{2+} Konzentration ($[Ca^{2+}]_i$). Ko-Immunopräzipitationen und Proximity Ligation Assays zeigten eine verstärkte Interaktion von PTEN mit TRPC6 in humanen PASMC und Mauslungen als Reaktion auf Hypoxie. In PASMC konnte sowohl für PTEN als auch für TRPC6 eine Translokation zu den Kaveolen unter hypoxischen Bedingungen nachgewiesen werden, welche wiederum durch die pharmakologische Inhibition der Rho-assoziiertem Protein Kinase ROCK blockiert wurde. Diese Inhibition verhindert die Interaktion von PTEN mit TRPC6, den Hypoxie-induzierten Ca^{2+} Anstieg und die HPV sowohl in PASMC als auch in murinen Lungen.

Fazit: Unsere Daten weisen auf ein PTEN vermitteltes Zusammenspiel zwischen ROCK und dem zytosolischen Ca^{2+} Signalweg bei der HPV hin. ROCK induziert dabei die Interaktion von PTEN mit TRPC6, die dann gemeinsam zu den Calveolen translozieren und dort den Ca^{2+} Einstrom und nachfolgend die Kontraktion der PASMC auslösen.

Affidavit

I, Adrienn Krauszman certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "The ROCK/PTEN/TRPC6 pathway in hypoxic pulmonary vasoconstriction". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The section on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) corresponds to the URM (s.o) and are answered by me. My contribution in the selected publication for this dissertation corresponds to those that are specified in the following joint declaration with the responsible person and supervisor.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date 16.09.2017   Signature

Detailed Declaration of Contribution

Adrienn Krauszman had the following share in the following publication:

Publication : Adrienn Krauszman, Tak W. Mak, PhD, Katalin Szaszi, MD PhD, Wolfgang M. Kuebler, MD, Role of phosphatase and tensin homolog (PTEN) in hypoxic pulmonary vasoconstriction, *Cardiovascular Research*, 2017.April (epub ahead of print).

Contribution in detail:

I, Adrienn Krauszman, made a literature research at the beginning of my doctoral studies and found that calcium entry to pulmonary artery smooth muscle cells (PASMC) triggers cell contraction in response to hypoxia. TRPC6 was found to be a critical cation channel in hypoxic pulmonary vasoconstriction (HPV) and it was previously researched in Dr. Kübler's lab. Further literature check revealed that in endothelial cells phosphatase and tensin homolog (PTEN) and TRPC6 interact and PTEN serves as a scaffold for TRPC6 enabling cell surface expression of the latter. I also found that PTEN can be regulated by Rho-kinase (ROCK). Based on these findings I hypothesized that ROCK and PTEN play an important role in HPV by regulating the calcium influx to PASMC via TRPC6.

I conducted all the work described in the research paper. I mastered the isolated perfused mouse lung model and utilized it for a series of experiments in cell-specific inducible knock out mice or C57 mice with pharmacological inhibitors. In this model, after intubation I inserted a cannula in the pulmonary artery of the mouse through the right ventricle, and another cannula to the left ventricle through the apex. Hanks balanced salt solution with 20% FBS was perfused through the lungs in a circulatory manner. Hypoxic pulmonary vasoconstriction was induced by ventilation with a gas mixture containing 1% O_2. In addition to assessing my own research question, this model provided me with multiple opportunities to collaborate with other labs, which resulted in several additional publications as a co-author.

I ran all western blots and immunoprecipitation assays described in the research paper, as well as for additional studies following a standard protcol and analysed the data from these experiments. I further developed a proximity ligation assay kit (Duolink) in our lab to test for the interaction of PTEN and TRPC6. In this assay PASMC were treated in hypoxia or normoxia, then fixed with 4% paraformaldehyde. Then primary antibodies against two different proteins were added. The next step was the addition of two special secondary antibodies against the species of the primaries with special oligonucleotides at the end. If the probes are in proximity, then a subsequent enzymatic step can ligate the probes and a rolling cycle amplification yields a fluorescent signal in case of interaction and no signal if the proteins are not interacting. The kit was validated by checking with two primary antibodies against the same protein, resulting in strong signal. Duolink revealed interaction of TRPC6 and PTEN in response to hypoxia. This interaction was also confirmed by co-immunoprecipitation experiments where samples were incubated with a primary antibody, then with beads to capture the antibody. Results were obtained by running western blots of the immunoprecipitated samples.

Intracellular calcium concentration in PASMC was measured with fura-2-acetoxymethyl (Fura-2AM) fluorescent dye. Coverslips with cells were loaded with HBSS containing Fura-2AM. Coverslips were then mounted in a heated chamber and the samples were excited by $\lambda = 340$ and 380 nm and 510 nm emission wavelength was collected.

A Rho-kinase inhibitor, Y27632, was used in the isolated perfused mouse lung model, in calcium concentration measurements and in the interaction studies and this inhibited HPV, and the calcium concentration increase and the interaction of PTEN and TRPC6 in response to hypoxia was also blocked.

A special subdomain of the plasma membrane, called caveolae, was separated by sucrose gradient centrifugation. In these experiments the sample is mixed with 80% sucrose 1:1, following by a 30% and a 5% sucrose layer. This was then ultracentrifugated and 10 equal fractions were carefully taken. PTEN and TRPC6 localization was then checked by western blot and I found that both proteins are present in the caveolar fractions after hypoxia treatment, but not under normoxic conditions and not if the cells were treated with Y27632 prior to and during hypoxia treatment.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Excerpt of the Journal Summary List (ISI Web of KnowledgeSM)

Journal Data Filtered By: **Selected JCR Year: 2016** Selected Editions: SCIE,SSCI
Selected Categories: **"CARDIAC and CARDIOVASCULAR SYSTEMS"** Selected
Category Scheme: WoS
Gesamtanzahl: 126 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY	96,042	19.896	0.227390
2	EUROPEAN HEART JOURNAL	51,199	19.651	0.142910
3	CIRCULATION	165,641	19.309	0.245560
4	Nature Reviews Cardiology	4,406	14.299	0.018530
5	CIRCULATION RESEARCH	49,784	13.965	0.079890
6	JACC-Cardiovascular Imaging	6,895	10.189	0.027050
7	JACC-Cardiovascular Interventions	8,512	8.841	0.035150
8	JACC-Heart Failure	2,047	8.493	0.012210
9	PROGRESS IN CARDIOVASCULAR DISEASES	3,544	8.177	0.007690
10	Circulation-Cardiovascular Interventions	4,207	7.198	0.019610
11	JOURNAL OF HEART AND LUNG TRANSPLANTATION	9,754	7.114	0.023940
12	EUROPEAN JOURNAL OF HEART FAILURE	9,839	6.968	0.026570
13	JOURNAL OF THE AMERICAN SOCIETY OF ECHOCARDIOGRAPHY	11,343	6.852	0.018680
14	Circulation-Cardiovascular Imaging	4,472	6.803	0.019120
15	Circulation-Heart Failure	5,492	6.372	0.024870
16	INTERNATIONAL JOURNAL OF CARDIOLOGY	25,609	6.189	0.068070
17	HEART	16,722	6.059	0.036180
18	European Heart Journal-Cardiovascular Imaging	3,168	5.990	0.014390
19	CARDIOVASCULAR RESEARCH	21,894	5.878	0.030340
20	JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY	14,017	5.680	0.027280
21	JOURNAL OF CARDIOVASCULAR MAGNETIC RESONANCE	4,349	5.601	0.014950
22	Circulation-Arrhythmia and Electrophysiology	5,670	5.410	0.023710
23	BASIC RESEARCH IN CARDIOLOGY	3,770	5.306	0.007660

1

Selected JCR Year: 2016; Selected Categories: "CARDIAC and CARDIOVASCULAR SYSTEMS"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
24	EuroIntervention	4,910	5.165	0.017720
25	TRENDS IN CARDIOVASCULAR MEDICINE	2,528	4.964	0.004340
26	HEART RHYTHM	10,902	4.866	0.034340
27	Journal of the American Heart Association	5,750	4.863	0.030730
28	Clinical Research in Cardiology	2,738	4.760	0.007490
29	Cardiovascular Diabetology	3,960	4.752	0.010500
30	Circulation-Cardiovascular Genetics	2,923	4.743	0.012380
31	EUROPACE	7,911	4.530	0.024360
32	Circulation-Cardiovascular Quality and Outcomes	3,884	4.524	0.017980
33	REVISTA ESPANOLA DE CARDIOLOGIA	3,150	4.485	0.005180
34	JOURNAL OF THORACIC AND CARDIOVASCULAR SURGERY	26,652	4.446	0.046950
35	AMERICAN HEART JOURNAL	21,638	4.436	0.035890
36	CANADIAN JOURNAL OF CARDIOLOGY	5,332	4.403	0.016290
37	ATHEROSCLEROSIS	22,724	4.239	0.041430
38	JOURNAL OF NUCLEAR CARDIOLOGY	3,021	3.930	0.003920
39	JOURNAL OF CARDIAC FAILURE	4,983	3.765	0.011520
40	EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY	14,568	3.759	0.028700
41	ANNALS OF THORACIC SURGERY	34,974	3.700	0.049810
42	NUTRITION METABOLISM AND CARDIOVASCULAR DISEASES	4,638	3.679	0.010720
43	European Journal of Preventive Cardiology	2,498	3.606	0.009640
44	CIRCULATION JOURNAL	9,700	3.544	0.020750
45	HEART FAILURE REVIEWS	2,076	3.481	0.004950
46	HEART AND VESSELS	2,167	3.434	0.003080
47	AMERICAN JOURNAL OF CARDIOLOGY	37,730	3.398	0.055360
48	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	30,912	3.348	0.031430
49	RESPIRATORY MEDICINE	10,267	3.217	0.016750

Selected JCR Year: 2016; Selected Categories: "CARDIAC and CARDIOVASCULAR SYSTEMS"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
50	Journal of Cardiovascular Computed Tomography	1,331	3.185	0.004220
51	JOURNAL OF CARDIOVASCULAR ELECTROPHYSIOLOGY	7,384	3.068	0.015470
52	JOURNAL OF CARDIOVASCULAR PHARMACOLOGY AND THERAPEUTICS	1,127	3.000	0.002960
53	CARDIOVASCULAR DRUGS AND THERAPY	1,821	2.820	0.002830
54	American Journal of Cardiovascular Drugs	978	2.768	0.002050
55	European Journal of Cardiovascular Nursing	1,311	2.763	0.002810
56	CLINICAL CARDIOLOGY	3,615	2.757	0.006190
57	Journal of Cardiology	2,452	2.732	0.005780
58	Cardiovascular Toxicology	1,038	2.712	0.001760
59	CATHETERIZATION AND CARDIOVASCULAR INTERVENTIONS	8,581	2.602	0.019320
60	Cardiology in Review	971	2.519	0.002260
61	Cardiovascular Therapeutics	1,024	2.478	0.002990
62	CARDIOVASCULAR PATHOLOGY	1,871	2.359	0.003630
63	Archives of Cardiovascular Diseases	1,228	2.331	0.003840
64	Journal of Cardiovascular Translational Research	1,412	2.319	0.005200
65	JOURNAL OF CARDIOVASCULAR PHARMACOLOGY	5,970	2.247	0.006310
66	CURRENT PROBLEMS IN CARDIOLOGY	468	2.217	0.000630
67	CARDIOVASCULAR AND INTERVENTIONAL RADIOLOGY	4,859	2.191	0.008890
68	JOURNAL OF CARDIOVASCULAR SURGERY	2,137	2.179	0.004230
69	Pulmonary Circulation	946	2.178	0.004200
70	Heart Failure Clinics	760	2.124	0.002490
71	Journal of Cardiovascular Nursing	1,579	2.105	0.002820
72	CURRENT OPINION IN CARDIOLOGY	1,953	2.080	0.004660
73	Current Cardiology Reports	1,338	2.058	0.004720
74	Heart Lung and Circulation	2,007	2.028	0.005900
75	International Heart Journal	1,294	2.017	0.002380

Selected JCR Year: 2016; Selected Categories: "CARDIAC and CARDIOVASCULAR SYSTEMS"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
76	INTERNATIONAL JOURNAL OF CARDIOVASCULAR IMAGING	2,742	1.896	0.007940
77	Netherlands Heart Journal	1,013	1.894	0.001950
78	JOURNAL OF INTERVENTIONAL CARDIOLOGY	1,235	1.880	0.002700
79	Interactive Cardiovascular and Thoracic Surgery	4,872	1.857	0.012470
80	ANNALS OF NONINVASIVE ELECTROCARDIOLOGY	1,188	1.852	0.002160
81	CardioRenal Medicine	305	1.844	0.001130
82	BMC Cardiovascular Disorders	2,089	1.832	0.006040
83	JOURNAL OF INTERVENTIONAL CARDIAC ELECTROPHYSIOLOGY	1,506	1.826	0.004120
84	CORONARY ARTERY DISEASE	1,831	1.823	0.003150
85	Journal of Cardiopulmonary Rehabilitation and Prevention	1,439	1.815	0.002350
86	Journal of Geriatric Cardiology	558	1.806	0.001860
87	CARDIOLOGY	2,382	1.742	0.003910
88	JOURNAL OF CARDIOTHORACIC AND VASCULAR ANESTHESIA	3,863	1.699	0.007030
89	PEDIATRIC CARDIOLOGY	4,014	1.688	0.009080
90	HEART & LUNG	2,193	1.657	0.003150
91	Journal of Cardiovascular Medicine	1,477	1.613	0.003150
92	Cardiovascular Ultrasound	919	1.598	0.001880
93	Annals of Thoracic Medicine	576	1.529	0.001130
94	JOURNAL OF ELECTROCARDIOLOGY	2,577	1.514	0.004220
95	PACE-PACING AND CLINICAL ELECTROPHYSIOLOGY	5,454	1.486	0.008810
96	THORACIC AND CARDIOVASCULAR SURGEON	1,681	1.424	0.003000
97	JOURNAL OF INVASIVE CARDIOLOGY	1,962	1.399	0.003960
98	CARDIOLOGY CLINICS	847	1.360	0.001450
99	Hellenic Journal of Cardiology	718	1.343	0.001080
100	Kardiologia Polska	1,257	1.341	0.002210

Selected JCR Year: 2016; Selected Categories: "CARDIAC and CARDIOVASCULAR SYSTEMS"

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
101	ECHOCARDIOGRAPHY-A JOURNAL OF CARDIOVASCULAR ULTRASOUND AND ALLIED TECHNIQUES	2,883	1.314	0.006370
102	Congenital Heart Disease	1,018	1.278	0.003410
103	Cardiology Journal	1,027	1.256	0.002860
104	Korean Circulation Journal	762	1.252	0.001780
105	Revista Portuguesa de Cardiologia	756	1.195	0.001300
106	Anatolian Journal of Cardiology	985	1.190	0.001700
107	Arquivos Brasileiros de Cardiologia	2,356	1.186	0.003100
108	PERFUSION-UK	1,003	1.134	0.001720
109	Journal of Cardiothoracic Surgery	1,459	1.101	0.004380
110	SCANDINAVIAN CARDIOVASCULAR JOURNAL	771	1.089	0.001350
111	Cardiovascular Engineering and Technology	282	1.064	0.001010
112	Cardiovascular Journal of Africa	600	0.967	0.001630
113	Reviews in Cardiovascular Medicine	291	0.929	0.000520
114	Postepy w Kardiologii Interwencyjnej	171	0.917	0.000370
115	CARDIOLOGY IN THE YOUNG	1,982	0.905	0.003880
116	EUROPEAN HEART JOURNAL SUPPLEMENTS	615	0.896	0.000340
117	TEXAS HEART INSTITUTE JOURNAL	1,709	0.895	0.002830
118	ACTA CARDIOLOGICA	913	0.808	0.001380
119	HERZ	967	0.776	0.001630
120	MINERVA CARDIOANGIOLOGICA	351	0.695	0.000460
121	Annals of Thoracic and Cardiovascular Surgery	1,045	0.674	0.001790
122	Revista Brasileira de Cirurgia Cardiovascular	497	0.601	0.000860
123	JOURNAL OF CARDIAC SURGERY	1,431	0.518	0.003090
124	HEART SURGERY FORUM	503	0.355	0.000770
125	Acta Cardiologica Sinica	183	0.342	0.000280
126	KARDIOLOGIYA	373	0.189	0.000210

Selected JCR Year: 2016; Selected Categories: "CARDIAC and CARDIOVASCULAR SYSTEMS"

Role of phosphatase and tensin homolog in hypoxic pulmonary vasoconstriction

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Aims	Hypoxic pulmonary vasoconstriction (HPV) redistributes blood flow from poorly ventilated to better aerated areas in the lung, thereby optimizing ventilation-perfusion ratio (V/Q). Pulmonary artery smooth muscle cell (PASMC) contraction in response to hypoxia is triggered by Ca^{2+} influx via transient receptor potential canonical 6 (TRPC6) cation channels that have translocated to caveolae in the plasma membrane. Since *phosphatase and tensin homolog* (PTEN) was suggested to regulate TRPC6 in endothelial cells, we aimed to define its role in the hypoxic response of PASMCs and as a putative mediator of HPV.
Methods and results	In isolated perfused mouse lungs, smooth muscle specific PTEN deficiency attenuated pulmonary vasoconstriction in response to hypoxia but not to angiotensin II (Ang II). Analogously, siRNA-mediated knock down of PTEN in human PASMC inhibited the hypoxia-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). Co-immunoprecipitation and proximity ligation assays revealed increased interaction of PTEN with TRPC6 in human PASMC and murine lungs in response to hypoxia. In hypoxic PASMC, both PTEN and TRPC6 translocated to caveolae, and this response was blocked by pharmacological inhibition of Rho-associated protein kinase (ROCK) which in parallel prevented PTEN-TRPC6 interaction, hypoxia-induced $[Ca^{2+}]_i$ increase, and HPV in PASMC and murine lungs, respectively.
Conclusion	Our data indicate a novel interplay between ROCK and $[Ca^{2+}]_i$ signalling in HPV via PTEN, in that ROCK mediates interaction of PTEN and TRPC6 which then conjointly translocate to caveolae allowing for Ca^{2+} influx into and subsequent contraction of PASMC.
Keywords	Hypoxia • Phosphatase and tensin homolog (PTEN) • Transient receptor potential canonical 6 (TRPC6) • Pulmonary artery smooth muscle cells (PASMC) • Rho kinase (ROCK)

1. Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a physiological response to alveolar hypoxia which redistributes pulmonary blood flow from poorly aerated lung regions to better ventilated lung segments by an active process of local vasoconstriction. Impaired HPV, as seen in a variety of lung diseases including pulmonary hypertension (PH), pneumonia, or sepsis, results in submaximal oxygenation of arterial blood and limits oxygen supply to systemic organs.[1–3] Global hypoxia, as seen at high altitude or during chronic hypoxic lung diseases such as chronic obstructive pulmonary disease, sleep apnea, or lung fibrosis may, on the other hand, cause generalized and sustained pulmonary vasoconstriction leading to vascular remodelling, right ventricular hypertrophy, and ultimately cor

pulmonale.[4] Although various regulatory pathways involved in HPV have been identified, considerable gaps in our knowledge and understanding remain, and a unifying concept of the underlying signalling pathways has not yet emerged. Thus, HPV continues to be an area of intense biomedical research with important clinical and therapeutic relevance.[5,6]

Increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) act as second messenger signal triggering pulmonary artery smooth muscle cell (PASMC) contraction in response to hypoxia.[7–10] In PASMC, hypoxia causes recruitment of transient receptor potential canonical 6 (TRPC6) to caveolae[11] where it is considered to initiate the PASMC $[Ca^{2+}]_i$ response.[12] However, the mechanisms regulating TRPC6 activation and its recruitment to, or trafficking within, the plasma membrane in hypoxia are poorly understood. In endothelial cells (ECs), *phosphatase and tensin*

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homolog (PTEN), a lipid and protein phosphatase, has been shown to serve as a scaffold for TRPC6, enabling cell surface expression of the channel and subsequent Ca^{2+} entry. Notably, this effect of PTEN is independent of its phosphatase activity, but mediated through direct interaction of PTEN with TRPC6 via its PDZ-binding domain.[13]

In addition to Ca^{2+} signalling via TRPC6, HPV requires activation of Rho kinase (ROCK).[14,15] which is considered to act predominantly through its Ca^{2+} sensitizing effects. Consistent with this notion, we recently showed that EC-derived sphingosine-1-phosphate (S1P) mediates HPV in a ROCK-dependent manner, and triggers the translocation of TRPC6 to caveolae and its activation in PASMC.[16]

Importantly, PTEN activity has previously been reported to be regulated by RhoA and its effector ROCK in pre-osteoblasts, as siRNA-based silencing of RhoA or inhibition of ROCK by Y27632 both decrease PTEN activity. This effect has been proposed to be dependent on direct interaction of PTEN with ROCK as shown by co-immunoprecipitation.[17] Rho furthermore regulates PTEN's localization in chemotaxing neutrophils via phosphorylation and interaction of the two proteins.[18] Activation of the Rho-ROCK-PTEN pathway has also been shown to mediate EC permeability changes in response to S1P.[19]

Based on the reported roles and regulation of PTEN in other cell types, we hypothesized that PTEN may play a critical role in HPV, and provide a key missing link between Rho/ROCK signalling and TRPC6-mediated Ca^{2+} influx into PASMC. Here we show that PTEN and TRPC6 interact in PASMC in response to hypoxia or S1P in a ROCK dependent manner, and that this interaction is required for their translocation to caveolae and the subsequent increase in PASMC $[Ca^{2+}]_i$ that result in smooth muscle contraction and HPV.

2. Methods

This is a short version of the protocols used, further details are provided in the Supplementary Material.

2.1 Animals

Male C57/Bl6 mice (25–30 g) were obtained from Charles River (Canada); mice with a conditional deletion in PTEN in smooth muscle cells (SMCs) were generated by crossing PTEN$^{flox/flox}$ (from Dr Tak W. Mak)[20] with tet-O-Cre and SMA-rtTA strains (kindly provided by Dr Dean Sheppard; UCSF)[21] to yield tet-O-Cre$^{tg/-}$;SMA-rtTA$^{tg/-}$; PTEN$^{fl/fl}$ (SMC-specific knock out mice) and tet-O-Cre$^{tg/-}$;SMA-rtTA$^{tg/-}$;PTEN$^{WT/WT}$ (control). All animals received care in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication 8th edition, 2011). Experiments were approved by the Animal Care and Use Committee of St Michael's Hospital. PTEN knockout mice and the control group received doxycycline in food (2 g/kg, TD.140011, Envigo, Madison, WI) for 1 week prior to experiments to induce gene knock out.

2.2 Isolated perfused mouse lung

Isolated perfused mouse lung (IPL) were prepared from male C57Bl6, tet-O-Cre$^{tg/-}$;SMA-rtTA$^{tg/-}$:PTEN$^{fl/fl}$, and tet-O-Cre$^{tg/-}$; SMA-rtTA$^{tg/-}$;PTEN$^{WT/WT}$ mice as previously described in.[22] In brief, mice were anaesthetized with 250 mg/kg avertin via IP injection, depth of anaesthesia was confirmed by toe pinch. Following anaesthesia and tracheotomy the heart was catheterized and pulmonary artery pressure (PAP) was recorded continuously. Lungs were only included in the study if perfusion pressure was stable and below 20 cm H_2O during the initial 10 min of baseline perfusion and if lungs showed no macroscopic signs of hemorrhage, atelectasis, or edema.

2.3 Cell culture

PASMCs, culture media kits (SmGM-2 BulletKit, CC-3182) and subculture supplies including HEPES buffered saline solution (CC-5024), trypsin/EDTA solution (CC-5012) and trypsin neutralizing solution (CC-5002) were purchased from Lonza (Clonetics PASMC, Lonza, Basel, Switzerland). Cells were cultured according to manufacturer's instructions and used within the first six passages of growth from three different batches.

2.4 Protein extraction

Cells were grown to confluence and treated as indicated in text, then lysed following standard protocols. Protein concentration was measured by BCA assay (Thermo Fischer Scientific, Waltham, MA) and samples were stored at -80 °C.

2.5 Hypoxia exposure *in vitro*

Experiments for hypoxia exposure of PASMC were performed in a custom-built hypoxia chamber. After 5 min of hypoxia cells were lysed following standard protocols and placed on ice immediately.

2.6 Western blotting

The following antibodies were used following standard western blotting procedures: TRPC6 1:1000—Alomone Lab, Jerusalem, Israel: ACC-017[23]; PTEN 1:1000—Cell Signaling, Boston, MA: 9552S[24]; GAPDH 1:1000—Santa Cruz, Dallas, TX: sc-25778; Caveolin-1 1:500—BD Biosciences, San Jose, CA: 610407.

2.7 Immunoprecipitation

One millilitre PASMC lysate was rotated with 20 µL Protein A/G beads (GE Healthcare, Mississauga, ON) for 30 min at 4 °C for pre-clearance. After centrifugation at 6000 rpm and 4 °C for 1 min the supernatant was collected, 1:100 anti-PTEN (Cell Signaling, Boston, MA: 9552S) was added to 750 µg total protein sample, and samples were rotated overnight at 4 °C. The next day, 25 µL of Protein A/G beads (GE Healthcare, Mississauga, ON) was added and samples were kept rotating for 1 h. Beads were washed thrice with lysis buffer and boiled with 30 µL Laemmli buffer (BioRad, Canada).

2.8 Ca^{2+} imaging in PASMC

Changes in PASMC $[Ca^{2+}]_i$ were measured as previously described.[16] PASMCs cultured on coverslips were loaded with HBSS containing 5 µmol/L fura-2-acetoxymethyl ester (fura-2AM) (Life Technologies, Carlsbad, CA) dissolved in Pluronic F-127 (20% solution in DMSO) (Life Technologies, Carlsbad, CA), then mounted in a heated chamber (Warner Industries; Saint-Laurent, QC, RC-21B + PH-2) at 37 °C. Fura-2 fluorescence was excited by monochromatic illumination (Polychrome V; TillPhotonics, Victor, NY) at λ = 340 and 380 nm and collected at an emission wavelength of 510 nm via a custom-built upright fluorescence microscope equipped with appropriate dichroic and emission filters and a digital camera. After background correction, the 340/380 ratio was calculated using TillVision 4.0 software (Till Photonics, Germany).

2.9 siRNA transfection

PASMC were seeded on 6-well plates. At 70% confluency cells were transfected with siRNA (On Target PTEN siRNA and non-targeting

control; Dharmacon, Ottawa, ON) using Effectene Transfection Reagent from Qiagen (Toronto, ON) according to manufacturer's instructions.

2.10 Immunofluorescence microscopy

Following exposure to normoxia or hypoxia, cells were fixed with paraformaldehyde and standard immunofluorescence staining protocols were performed.

2.11 Proximity ligation assay

Protein–protein interaction was assessed by proximity ligation assay (PLA) (DuoLink assay; Sigma Aldrich, Oakville, ON). Samples were prepared as described for immunofluorescence microscopy. After incubation with two primary antibodies from different species and directed against two putatively interacting proteins, two species-specific secondary antibodies directed against the different primary antibodies with complementary oligonucleotide sequences at their ends (PLA probes) were added. PLA probes in close proximity (<40 nm) were joined by enzymatic ligation, and the resulting signal was amplified with rolling cycle amplification and visualized using fluorescently labelled complementary oligonucleotides by confocal laser spinning disk microscopy. Raw, single plain images were quantified in ImageJ (National Institute of Health, USA).

2.12 Isolation of caveolar fractions by sucrose density gradient ultracentrifugation

Isolation of caveolar fractions from PASMC and probing for caveolar abundance of proteins of interest was performed as recently reported using sucrose gradient centrifugation.[16]

2.13 Isolation of PASMC from murine lungs

Under deep anaesthesia, the murine heart and lungs were carefully removed. The trachea, the vena cava and the left lungs were pinned down. The pulmonary artery (PA) was microsurgically separated from the adjacent vein and bronchi, and small pieces of the isolated artery were placed in a T25 flask with droplets of SMC medium containing an additional 15% FBS. These were cultured for 2 weeks before use.

2.14 Statistical analysis

Statistical analyses were performed with GraphPad Prism using Mann-Whitney U test for two independent groups, or One-way Anova for more than two groups. For analysis of multiple cells per isolation (*Figures 2C,D* and *5B*) a hierarchical linear model (with random effect, build nested terms) was used with SPSS Statistics. Data are shown as mean ± SEM, differences were considered significant (*) at $P < 0.05$.

3. Results

3.1 PTEN is required for intact HPV

To test for a potential role of PTEN in HPV we generated SMC-specific PTEN knockout mice (SMC PTEN KO). Inducible deletion of PTEN from SMCs in mice was confirmed by western blot analysis of an SMC rich organ (intestine) and an organ containing minimal amounts of SMC (heart). PTEN levels were significantly reduced in the intestines of KO mice as compared with wild type (WT) controls while no significant
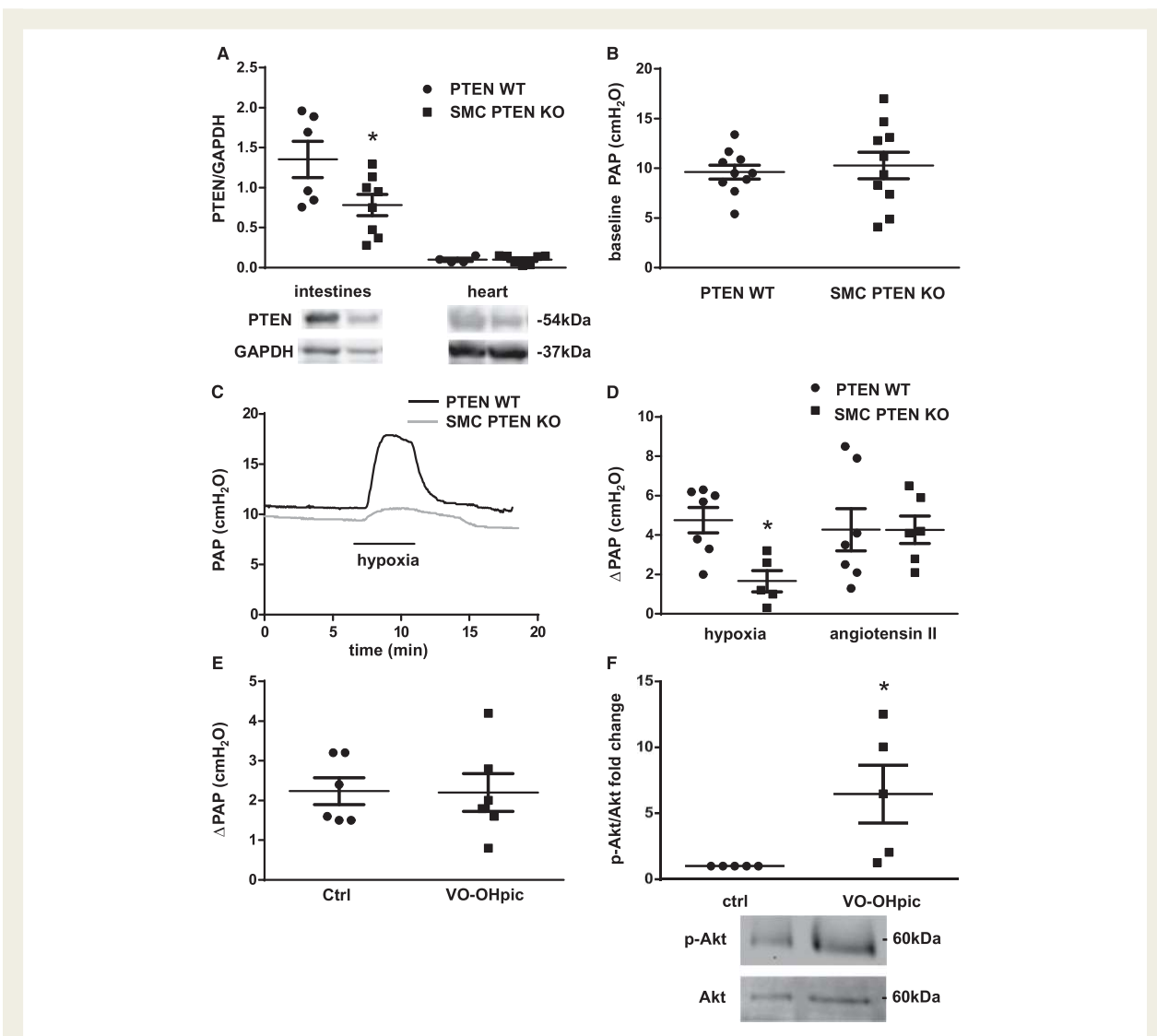
difference in PTEN expression was detectable in the heart (*Figure 1A*). Baseline perfusion pressures in isolated lungs did not differ between WT and SMC PTEN KO mice (*Figure 1B*). We next tested the vasoconstriction response to hypoxia and Ang II in the IPL model by measuring the PAP change in response to a switch from normoxic (21% O_2) to hypoxic (1% O_2) ventilation or bolus infusion of Ang II, respectively. Although WT lungs showed the characteristic pressure response to hypoxia, SMC PTEN KO lungs had a largely reduced HPV (*Figures 1C* and *D*). PTEN deficiency in SMCs did, however, not prevent vasoconstriction in response to Ang II (*Figure 1D*). Inhibition of PTEN's phosphatase activity by the vanadate compound VO-OHpic did also not attenuate HPV (*Figure 1E*), suggesting that the role of PTEN in HPV is independent of its phosphatase activity. VO-OHpic increased Akt phosphorylation (*Figure 1F*), demonstrating that the applied dose was effective in inhibiting PTEN[25–27] in the isolated lung preparation.

3.2 PTEN knockdown attenuates hypoxia-induced PASMC contraction and $[Ca^{2+}]_i$ increase

Ca^{2+} entry into PASMCs is a known prerequisite for HPV. Hence, we next tested whether loss of PTEN in PASMC may attenuate PASMC $[Ca^{2+}]_i$ signalling in response to hypoxia. PASMCs were transfected with PTEN-specific or control siRNA (siPTEN or siCtrl, respectively), and effective silencing of PTEN by ~50% was confirmed by Western Blot (*Figure 2A*). PASMC treated with scrambled siRNA showed the characteristic $[Ca^{2+}]_i$ increase in response to hypoxia; yet, silencing PTEN with siPTEN significantly reduced this response (*Figure 2B* and *C*). A similar effect was observed in murine PASMC in that cells isolated from SMC PTEN KO mice had a reduced $[Ca^{2+}]_i$ response to hypoxia compared with cells from WT animals (*Figure 2D*).

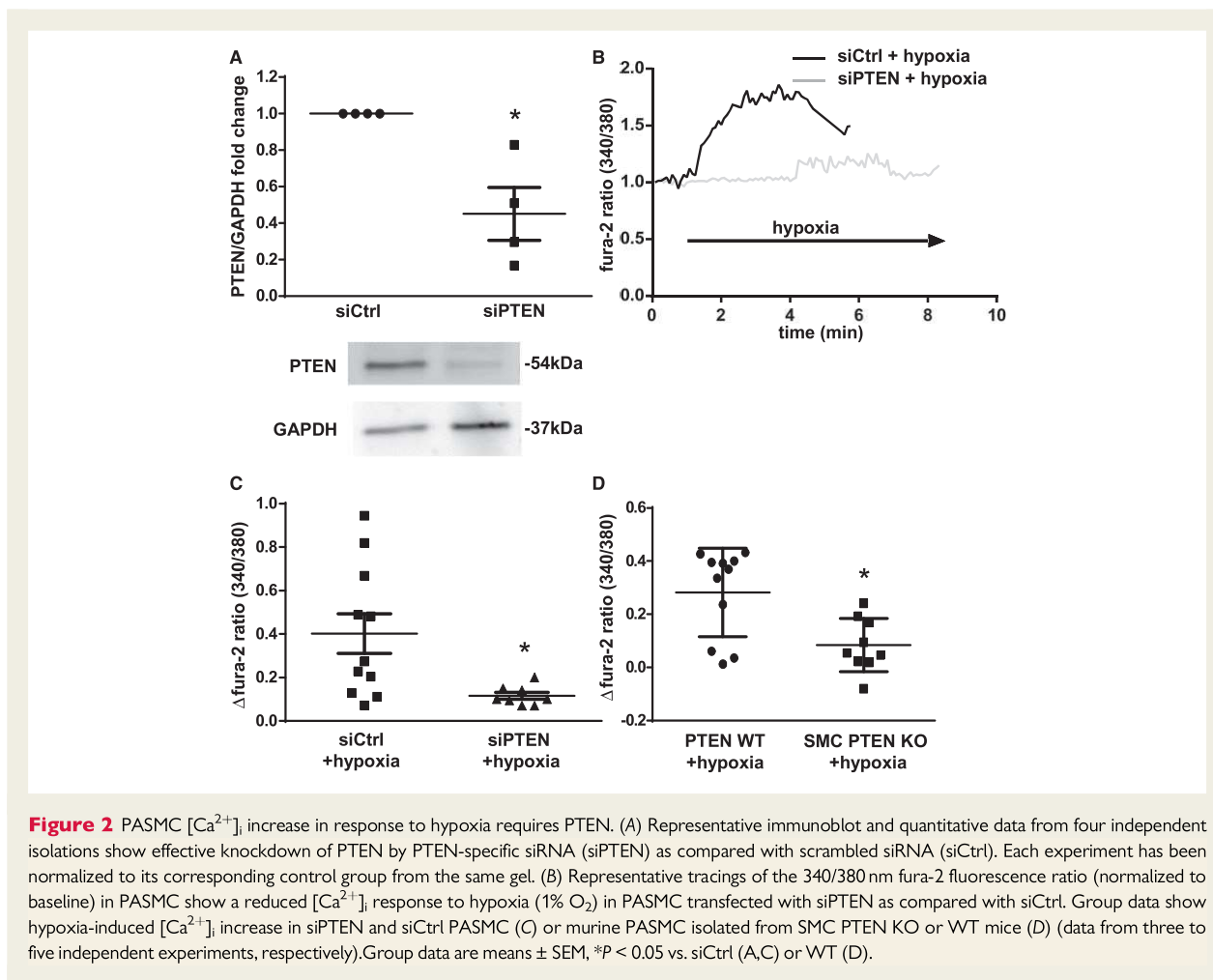## 3.3 Hypoxia and S1P induce PTEN interaction with TRPC6 through ROCK

In ECs PTEN has been shown to interact with TRPC6, a cation channel that plays a key role in HPV. To probe for a similar interaction of PTEN with TRPC6 in PASMCs in response to either hypoxia or S1P, a central mediator of HPV, we immunoprecipitated PTEN from PASMC following either 5 min of hypoxia (in the presence or absence of the ROCK inhibitor Y27632) or normoxia, or S1P treatment (*Figure 3A*), and blotted the immunoprecipitates for both PTEN and TRPC6. Co-immunoprecipitation showed increased interaction of PTEN with TRPC6 in PASMC stimulated with either hypoxia or S1P as compared to normoxic controls. As TRPC6 and PTEN are both regulated by ROCK,[17,19,28] we probed for the effects of the ROCK inhibitor Y27632 on the interaction of TRPC6 with PTEN. Y27632 blunted the hypoxia-induced interaction, indicating a regulatory role of ROCK that will be followed up further below. A similar increase in TRPC6/PTEN interaction was detected in whole lung lysates (*Figure 3B*) of IPLs exposed to hypoxia for 3 min as compared with normoxic controls. Increased interaction of PTEN with TRPC6 in PASMC in response to hypoxia or S1P was also confirmed by PLA (*Figure 3C* and *D*) where red puncta indicate interaction of the 2 proteins while nuclei were stained with DAPI (blue). Y27632 again blocked TRPC6/PTEN interaction in response to hypoxia or S1P, while lysophosphatidic acid (LPA), a known ROCK activator, recapitulated the effects of S1P and hypoxia, thus corroborating a regulatory role of ROCK in the interaction of PTEN with TRPC6.

Figure 1 HPV requires SMC PTEN. (*A*) Representative immunoblots and quantitative data (*n* = 4–8) show that SMC PTEN KO reduced PTEN expression in SMC-rich intestines, but not in the heart. (*B*) Quantitative data shows that baseline pressures are similar in IPL of KO animals compared with WT controls (*n* = 10 each). (*C*) Representative tracings of PAP in IPL experiments show attenuated vasoconstriction in response to hypoxia (1% O₂) in lungs of SMC PTEN KO mice compared with WT. (*D*) Group data show attenuated PAP increase (ΔPAP) 5 min after start of hypoxia but not in response to Ang II (1 µg bolus for 5 min) in SMC PTEN KO mice; (*n* = 5–6, respectively) compared with WT mice (*n* = 7 both). (*E*) VO-OHpic (10 µMol/L) did not affect the PAP response to hypoxia in isolated lungs of C57Bl/6 mice (*n* = 6 each). (*F*) Lungs were collected and snap-frozen after 30 min of perfusion in the presence or absence of VO-OHpic (10 µMol/L). Representative western blot of lung lysates and quantitative data show levels of p-Akt normalized to total Akt in the presence or absence of VO-OHpic (10 µMol/L) (*n* = 5). Group data are means ± SEM, *P < 0.05 vs. WT (A,D) or control (Ctrl; F).

3.4 PTEN mediates TRPC6 recruitment to caveolae in a ROCK-dependent manner

Caveolae are specialized membrane microdomains enriched in cholesterol, sphingolipids, and proteins that serve as signalling hubs in the plasma membrane. Caveolae play a critical role in Ca^{2+} influx via TRPC6 in pulmonary microvascular ECs[29] and PASMC,[11] in that they regulate the abundance of functional TRPC6 at the plasma membrane. To test whether PTEN and/or ROCK may play a role in TRPC6 translocation to caveolae, we prepared caveolar fractions by sucrose gradient centrifugation of PASMC lysates. When compared with normoxic controls (*Figure 4A, B,* and *D*), we found both PTEN and TRPC6 translocated to caveolae. As TRPC6/PTEN interaction had been inhibitable by Y27632, we next tested whether the trafficking of the two proteins to caveolae in response to hypoxia similarly required ROCK. Pretreatment of PASMC with Y27632 reduced the abundance of TRPC6 and PTEN in caveolae, indicating that the translocation of both proteins occurred in a ROCK-dependent manner (*Figure 4C* and *D*).

Figure 2 PASMC $[Ca^{2+}]_i$ increase in response to hypoxia requires PTEN. (*A*) Representative immunoblot and quantitative data from four independent isolations show effective knockdown of PTEN by PTEN-specific siRNA (siPTEN) as compared with scrambled siRNA (siCtrl). Each experiment has been normalized to its corresponding control group from the same gel. (*B*) Representative tracings of the 340/380 nm fura-2 fluorescence ratio (normalized to baseline) in PASMC show a reduced $[Ca^{2+}]_i$ response to hypoxia (1% O_2) in PASMC transfected with siPTEN as compared with siCtrl. Group data show hypoxia-induced $[Ca^{2+}]_i$ increase in siPTEN and siCtrl PASMC (*C*) or murine PASMC isolated from SMC PTEN KO or WT mice (*D*) (data from three to five independent experiments, respectively).Group data are means ± SEM, *$P < 0.05$ vs. siCtrl (A,C) or WT (D).

3.5 ROCK mediates PASMC $[Ca^{2+}]_i$ response to hypoxia and HPV

Analogous to the effects of PTEN knock-down, inhibition of ROCK also inhibited the hypoxia induced $[Ca^{2+}]_i$ increase in PASMC as measured by fura-2 ratiometric imaging (*Figure 5A* and *B*). Finally, ROCK inhibition attenuated HPV in IPLs (*Figure 5C*), consolidating the functional relevance of ROCK for the outlined signalling pathway.

4. Discussion

In this study, we identify a previously unrecognized regulatory role for PTEN in HPV. This role relates to its function in PASMC, as SMC-specific knockout of PTEN attenuated the characteristic pulmonary vasoconstrictive response to hypoxia, but not to the systemic vasoconstrictor Ang II. This role of PTEN in PASMC is independent of its phosphatase activity, as it was not blocked by the PTEN inhibitor VO-OHpic at effective pharmacological concentrations. Instead, PTEN may act as a scaffold for the polymodal cation channel TRPC6 which mediates PASMC Ca^{2+} entry in response to hypoxia,[12] as (i) hypoxia triggered the interaction of PTEN with TRPC6 in both intact lungs and isolated

PASMC, and (ii) PTEN knock-down blocked the hypoxia-induced $[Ca^{2+}]_i$ increase in PASMC. Both PTEN and TRPC6 translocated to caveolae in PASMC in response to hypoxia in a ROCK-dependent manner. ROCK inhibition in turn blocked PTEN-TRPC6 interaction, inhibited the PASMC $[Ca^{2+}]_i$ response to hypoxia, and attenuated HPV. Taken together, these findings identify a new signalling pathway for HPV in that hypoxia, and/or the hypoxia-generated mediator S1P, stimulate a ROCK-dependent interaction between PTEN and TRPC6 in PASMC which is required for the effective recruitment of TRPC6 to caveolae and the subsequent influx of Ca^{2+} that ultimately triggers PASMC contraction (*Figure 6*).

Ca^{2+} influx is the essential second messenger that triggers actin-myosin interaction and thus, the characteristic contraction of PASMCs in response to hypoxia. TRPC6 has been identified as a cation channel that is critical for both hypoxia-induced Ca^{2+} entry into PASMC and subsequent HPV in intact lungs.[12,30,31] Although TRPC6 by itself may not be the only cation channel mediating hypoxia-induced Ca^{2+} influx in PASMC, which has also been shown to involve voltage-gated Ca^{2+} channels,[32,33] TRPC6 activation has emerged as a key initiating event. Specifically, (i) TRPC6[16], yet not L-type channel inhibition[34,35] results in an almost complete loss of the PASMC Ca^{2+} response to hypoxia and
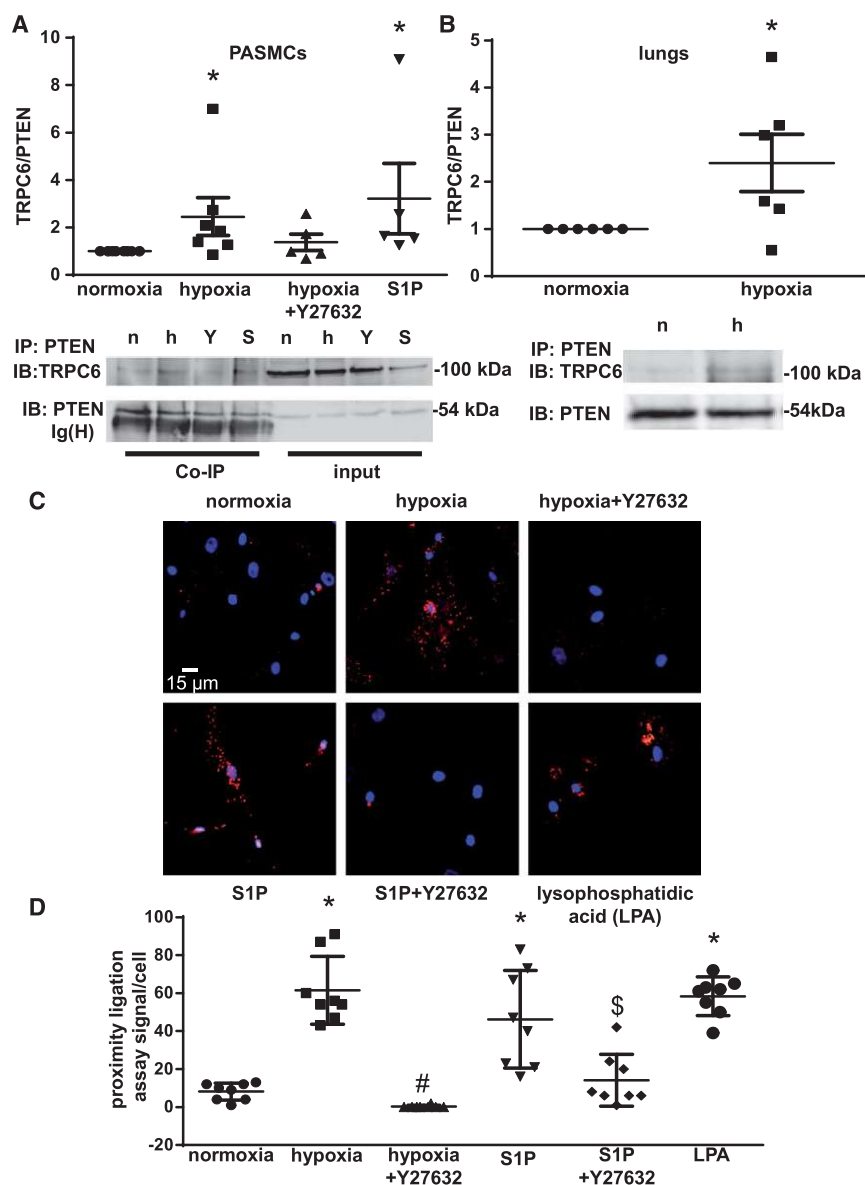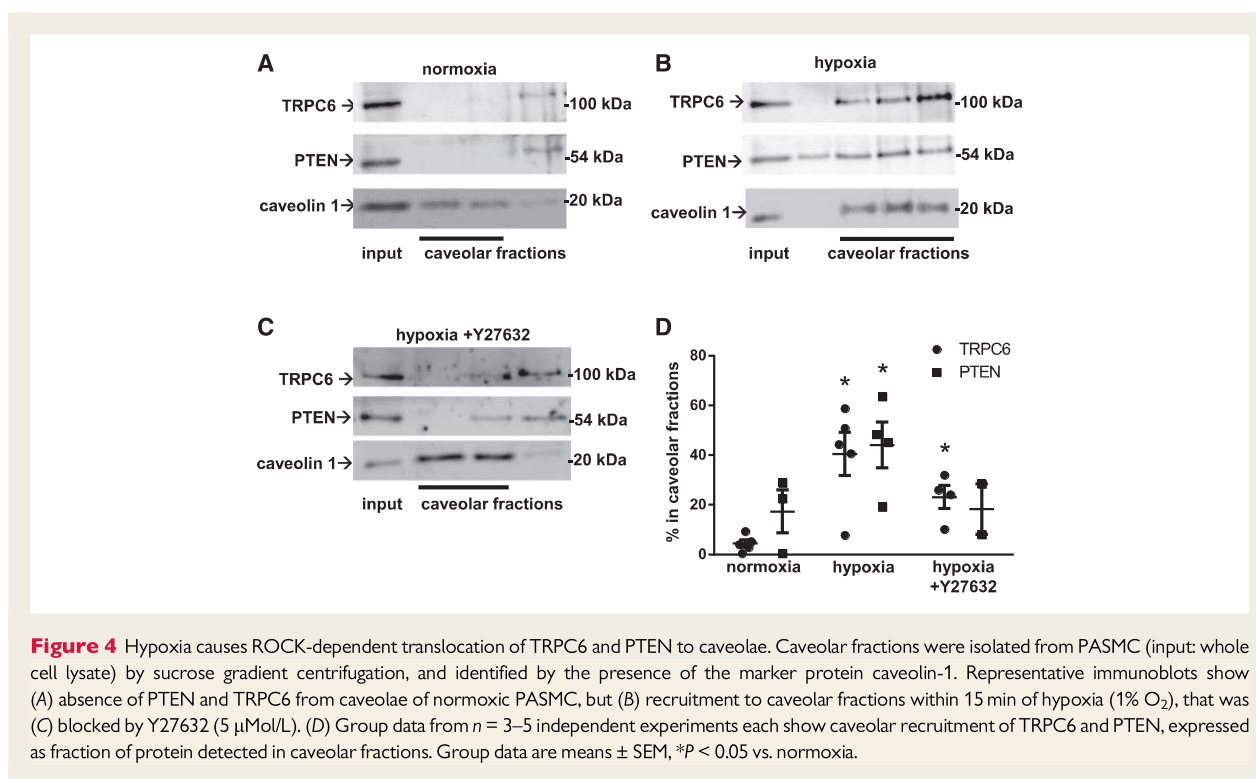
Figure 3 Hypoxia increases the interaction of PTEN with TRPC6 in a ROCK-dependent manner. (*A*) PASMCs were exposed to either normoxia (n), hypoxia (h; 1% O_2), or hypoxia in the presence of Y27632 (Y; 5µMol/L), or to S1P (S; 10µMol/L) for 5 min. Representative immunoblots show TRPC6 and PTEN expression in PASMC for whole cell lysate (input) and after immunoprecipitation for PTEN (Co-IP). Group data from $n = 5–7$ independent replicates showing TRPC6-over-PTEN ratio normalized to the normoxic control group from the same gel, demonstrate increased interaction of PTEN with TRPC6 in response to hypoxia and S1P, respectively. (*B*) Isolated lung were ventilated with normoxic or hypoxic (1% O_2) gas for 3 min, and tissue was snap-frozen and lysed. Representative immunoblots show TRPC6 and PTEN expression after immunoprecipitation for PTEN; group data from $n = 6$ replicates show quantification of TRPC6-over-PTEN ratio normalized to the normoxic control group from the same gel. (*C*) Representative images show PLA for the interaction between PTEN and TRPC6 in PASMC following exposure to either normoxia, hypoxia, or S1P(10µMol/L) for 5 min, in the presence or absence of Y27632 (5 µMol/L), or LPA (3 µMol/L) for 15 min. Red puncta indicate sites of interaction between PTEN and TRPC6, nuclei are counterstained in blue with DAPI. (*D*) Group data show quantification of the PLA from eight cells from three independent experiments each. Group data are means ± SEM, *$P < 0.05$ vs. normoxia, #$P < 0.05$ vs. hypoxia and $$P < 0.05$ vs. S1P.

Figure 4 Hypoxia causes ROCK-dependent translocation of TRPC6 and PTEN to caveolae. Caveolar fractions were isolated from PASMC (input: whole cell lysate) by sucrose gradient centrifugation, and identified by the presence of the marker protein caveolin-1. Representative immunoblots show (*A*) absence of PTEN and TRPC6 from caveolae of normoxic PASMC, but (*B*) recruitment to caveolar fractions within 15 min of hypoxia (1% O$_2$), that was (*C*) blocked by Y27632 (5 μMol/L). (*D*) Group data from $n = 3$–5 independent experiments each show caveolar recruitment of TRPC6 and PTEN, expressed as fraction of protein detected in caveolar fractions. Group data are means ± SEM, *P < 0.05 vs. normoxia.

(ii) TRPC6-mediated cation entry serves as initial trigger for activation of voltage-gated Ca^{2+} channels via membrane depolarization.[29] Following stimulation by hypoxia,[11] TRPC6 translocates to caveolae, specialized membrane microdomains that act as signalling hubs for outside-in-signalling and play key roles in regulating the abundance of ion channels at the plasma membrane and resulting ion fluxes, including those of Ca^{2+}.[11,16] However, the exact mechanisms underlying the hypoxia-induced recruitment of TRPC6 to caveolae remain unclear.

Recently, our group identified a critical role for endothelial-derived S1P as putative intercellular mediator during HPV, downstream of neutral sphingomyelinase activation.[16] In this work, we showed that S1P signalling is required for the translocation of TRPC6 to caveolae, and its activation to trigger Ca^{2+} influx into PASMC. We further demonstrated that S1P receptor-2 mediated activation of PLC is required for HPV, presumably acting via DAG synthesis and subsequent TRPC6 activation[36] that synergized with a parallel activation of the Rho/ROCK signalling pathway to elicit pulmonary vasoconstriction.[16] Notably, although the sphingolipid-mediated recruitment and activation of TRP channels in response to hypoxia is present in PASMC *per* se,[16] the HPV response in the intact lung requires additional input from the endothelium as a conducer of the hypoxic signal from the alveolar capillaries to the feeding arteries.

In ECs PTEN, a lipid and protein phosphatase protein, serves as a scaffold for TRPC6 after thrombin stimulation, enabling cell surface expression of the channel and subsequent Ca^{2+} entry. Notably, this effect of PTEN is independent of its phosphatase activity, but mediated through direct interaction of PTEN with TRPC6 via its PDZ-binding domain.[13] This is in line with our finding that inhibition of PTEN phosphatase activity did not affect HPV. Instead, we show by two different approaches,

namely co-immunoprecipitation in intact lungs and PLA in cultured PASMC that hypoxia as well as S1P trigger the interaction of PTEN with TRPC6. This finding is in line with previous data from ECs demonstrating that cell migration is regulated by S1P through activation of PTEN.[37] The relevance of the detected interaction between PTEN and TRPC6 is highlighted by the fact that siRNA-mediated knock-down of PTEN attenuated the characteristic increase in PASMC [Ca^{2+}]$_i$ in response to hypoxia, which had been identified to be mediated by TRPC6.[12] The importance of PTEN/TRPC6 interaction is furthermore underlined by our finding that SMC PTEN KO using a Cre-lox system attenuated HPV in *ex vivo* perfused mouse lungs. Loss of PTEN in SMC did; however, not attenuate vasoconstriction in response to Ang II. Although this finding does not preclude differences in the vasoconstriction response to other pharmacological agonists, it suggests that the role of PTEN in vasoconstriction may be specific for the response to hypoxia. Of relevance, SMC-specific PTEN deletion in mice has been demonstrated to result in age-dependent spontaneous PH.[38] We and others have shown that HPV is attenuated in mice with chronic hypoxic PH,[39,40] which is considered to contribute to systemic hypoxemia in PH patients.[41] In this study, however, experiments were conducted at an age of 8–12 weeks, i.e. prior to the onset of spontaneous PH, which was confirmed by the fact that lungs of SMC PTEN KO mice had similar baseline perfusion pressures as those of WT mice. Hence, while other key signalling molecules involved in HPV including TRPC6,[12] connexin 40,[42] CFTR,[16] or TRPV4[43] are typically also involved in the development of chronic hypoxic PH,[16,44–50] the described role of PTEN seems to present a rare case of a signalling pathway with opposing effects on HPV and PH. Importantly, this notion opens up the possibility that the inhibition of HPV (and thus, the hypoxemia caused by the resulting V/Q mismatch) are not exclusively caused by
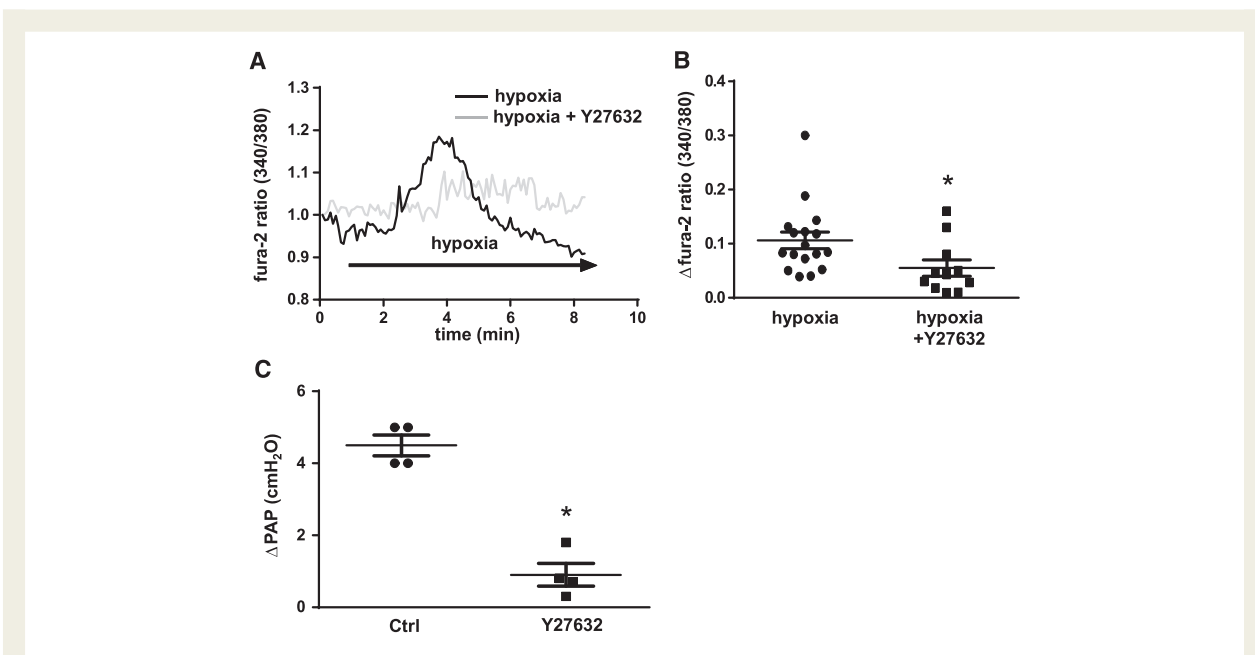
Figure 5 ROCK inhibition prevents $[Ca^{2+}]_i$ signalling and HPV response. (*A*) Representative tracings of the 340/380 nm fura-2 fluorescence ratio (normalized to baseline) in PASMC show reduced cytosolic $[Ca^{2+}]_i$ response to hypoxia (1% O_2) in PASMC in the presence of Y27632 (5 μMol/L). (*B*) Group data show effect of Y27632 on the hypoxia-induced $[Ca^{2+}]_i$ increase (data from 3 independent experiments each). (*C*) Group data show inhibition of the PAP increase (ΔPAP) by Y27632 in IPL 3 min after start of hypoxia (data from four independent experiments each). Group data are means ± SEM, *$P < 0.05$ vs. hypoxia (B) or control (Ctrl; C).
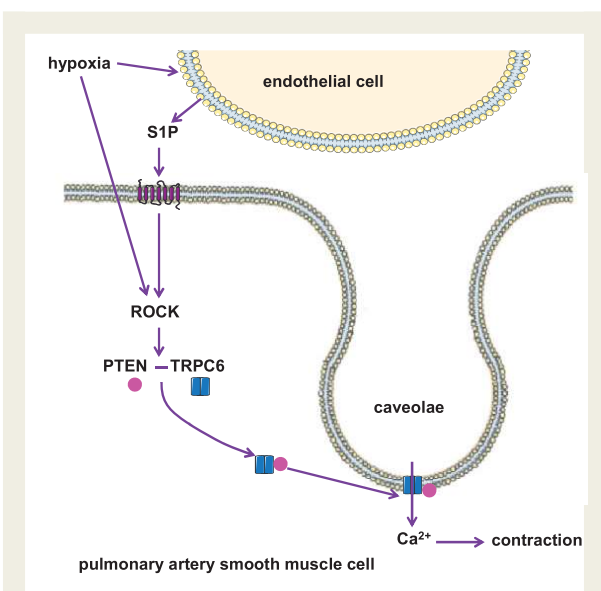
Figure 6 Schematic outline of the proposed role of PTEN in HPV: Hypoxia, either directly and/or via formation of S1P, activates ROCK in PASMC, which mediates the interaction between PTEN and TRPC6 and their translocation to caveolae, where TRPC6 facilitates Ca^{2+} entry and subsequent PASMC contraction.

an increased basal tone of the pulmonary resistance vessels which limits their ability to constrict further, but may in part result from activation of signalling pathways with opposing effects on HPV and PH.

Rho/ROCK signalling plays a central signalling role in SMC contraction, largely through its Ca^{2+} sensitizing role by inhibiting myosin light chain phosphatase.[15] Accordingly, Rho/ROCK signalling has been implicated in HPV,[14,51] a notion that was confirmed in this study in that HPV was markedly reduced by Y27632. In addition, however, Y27632 has been shown to directly inhibit hypoxia-induced increases in PASMC $[Ca^{2+}]_i$,[15] a finding that was again confirmed in this study, and that indicates that Rho/ROCK signalling may, in addition to its Ca^{2+}-sensitizing effects, act upstream of TRPC6-mediated Ca^{2+} entry in hypoxic PASMC. The latter notion is consistent with previous data from primary podocytes in which Rho-mediated PLCε stimulation was shown to activate TRPC6.[28]

In line with the newly identified role of PTEN in HPV and its previously reported regulation by ROCK,[16,19] we found inhibition of ROCK to prevent the interaction of TRPC6 with PTEN and their translocation to caveolae. The mechanism, however, by which ROCK mediates the interaction and caveolar recruitment of PTEN and TRPC6 in hypoxia remains to be resolved. Notably, dephosphorylated PTEN was shown to recruit to a protein complex to the plasma membrane whereas phosphorylation of the molecule prevented this response.[52,53] Hence, PTEN may become indirectly dephosphorylated in a ROCK-dependent manner, as previously shown for neuronal cells[54,55] through a pathway that has not yet been identified. Dephosphorylated PTEN may then facilitate, through direct protein–protein interaction, the recruitment of TRPC6 to caveolae. However, as co-immunoprecipitation as well as proximity

ligation and caveolar recruitment assays using specific phospho- and non-phospho-PTEN antibodies failed to yield reproducible results (data not shown), presumably due to limited antibody specificity, the exact mechanism by which ROCK mediates PTEN-TRPC6 interaction must remain speculative at the present stage.

In conclusion, we report here a new critical role of PTEN in hypoxia-induced vasoconstriction and Ca^{2+} signalling in PASMC. In response to hypoxia or S1P, PTEN interacts and translocates with TRPC6 into caveolae in a ROCK-dependent manner, thus highlighting the intricate interdependence between Ca^{2+} and Rho/ROCK signalling in HPV.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Author contributions

A.K., K.S., and W.M.K., designed the study and wrote the article. T.M. provided key materials. All authors interpreted the results and approved the final version of the article.

Conflict of interest: none declared.

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Publications

1. **Adrienn Krauszman**, Tak Mak, Katalin Szaszi, Wolfgang M. Kuebler. Role of phosphatase and tensin homolog (PTEN) in hypoxic pulmonary vasoconstriction. Cardiovasc Res. 2017 Apr 18. [Epub ahead of print]

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Conference presentations

1. Adrienn Krauszman, Dean Sheppard, Tak Mak, Wolfgang M. Kuebler (2016, April). Role of PTEN in hypoxic pulmonary vasoconstriction. Poster presentation at Experimental Biology 2016, San Diego

2. Adrienn Krauszman, Dean Sheppard, Tak Mak, Wolfgang M. Kuebler (2015, June). Signaling pathways in hypoxic pulmonary vasoconstriction. Oral presentation at the 2015 15th Annual Research Day in Respirology, Toronto

3. Adrienn Krauszman, Dean Sheppard, Tak Mak, Wolfgang M. Kuebler (2015, March). Role of PTEN in hypoxic pulmonary vasoconstriction. Poster presentation at Experimental Biology 2015, Boston

4. Adrienn Krauszman, Wolfgang M. Kuebler (2014, November). Role of PTEN in hypoxic pulmonary vasoconstriction. Poster presentation at the 2014 RTC Research Day, Toronto

5. Adrienn Krauszman, Wolfgang M. Kuebler (2014, June). Regulation of pulmonary vasoconstriction by hypoxia and sphingolipids. Poster presentation at the 2014 14th Annual Research Day in Respirology, Toronto

Acknowledgements

I would like to thank my supervisor, Wolfgang M. Kübler, for the opportunity to conduct research in his lab. He is the best supervisor I could've imagined with great ideas, fully equipped lab, and amazing connections. He guided me through the tough periods and gave me ideas to start my project but also gave me freedom to follow my hypotheses. I have to also thank Katalin Szászi and András Kapus for their guidance in the lab and also in my new life in Toronto, far away from home and everyone I know. I have to thank for the members of the core facility, especially Caterina DiCiano Oliveira, for their help with the equipment, and also the members of the Vivarium for their help with mouse transfers and breeding.

I would like to thank my collaborators for giving me the opportunity to practice my skills and for their trust with precious experiments.

I would like to thank my labmates in Toronto and Berlin, who quickly became really good friends, for their help in the lab and in real life. Without their friendship it would have been a much harder few years. Of course I have to thank my friends in Hungary for supporting me and being there for me even from such distance.

And last, but not least I want to thank my family for their support, during this long journey.