Aberrant Chloride Intracellular Channel 4 Expression Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension

Running title: Wojciak-Stothard et al.; CLIC4 and endothelial dysfunction in PAH

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Abstract

**Background**—Chloride intracellular channel 4 (CLIC4) is highly expressed in the endothelium of remodelled pulmonary vessels and plexiform lesions of patients with pulmonary arterial hypertension (PAH). CLIC4 regulates vasculogenesis through endothelial tube formation. Aberrant CLIC4 expression may contribute to the vascular pathology of PAH.

**Methods and Results**—CLIC4 protein expression was increased in plasma and blood-derived endothelial cells from patients with idiopathic PAH (IPAH) and in the pulmonary vascular endothelium of 3 rat models of pulmonary hypertension. CLIC4 gene deletion markedly attenuated the development of chronic hypoxia-induced pulmonary hypertension in mice. Adenoviral overexpression of CLIC4 in cultured human pulmonary artery endothelial cells compromised pulmonary endothelial barrier function and enhanced their survival and angiogenic capacity, while CLIC4 shRNA had an inhibitory effect. Similarly, inhibition of CLIC4 expression in blood-derived endothelial cells from patients with IPAH attenuated the abnormal angiogenic behaviour that characterises these cells. The mechanism of CLIC4 effects involves p65-mediated activation of nuclear factor-κB, followed by stabilisation of hypoxia-inducible factor-1α and increased downstream production of vascular endothelial growth factor and endothelin-1.

**Conclusions**—Increased CLIC4 expression is an early manifestation and mediator of endothelial dysfunction in pulmonary hypertension.

**Key words**: angiogenesis, endothelium, endothelin, hypoxia, hypertension, pulmonary
Introduction

Chloride intracellular channel 4 (CLIC4) is a member of a family of six highly conserved redox-sensitive proteins (CLIC1-6) homologous to glutathione transferases\(^1\). In its soluble form, CLIC4 is essential for normal vasculogenesis through endothelial tubulogenesis\(^2,3\). Knockout of the murine CLIC4 gene results in reduced collateral vessel formation \textit{in vivo}, associated with a reduction in ischemia-induced expression of hypoxia inducible factor-1\(\alpha\) (HIF-1\(\alpha\)) and downstream targets, such as (VEGF) and angiopoietin-2\(^4\).

CLIC4 is highly expressed in the lungs of patients with pulmonary arterial hypertension (PAH), compared with healthy lungs\(^5\). Increased CLIC4 expression is evident in the vascular endothelium and, in particular, in the occlusive and plexiform lesions that result from endothelial cell proliferation, impaired apoptosis and disorganised angiogenesis that characterise the disease\(^5\). The expression of CLIC4 is influenced by reactive oxygen species (ROS), DNA damage and cytokines, such as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and transforming growth factor-\(\beta\) (TGF-\(\beta\))\(^7,8\). CLIC4 is an integral component of TGF-\(\beta\) signalling through prevention of the dephosphorylation of phospho-Smad2 and 3 in the nucleus, as well as several other pathways that have been implicated in the pathogenesis of PAH, including TNF-\(\alpha\), bone morphogenetic protein (BMP) and Rho signalling\(^9,10\).

We hypothesized that aberrant CLIC4 expression may contribute to pulmonary endothelial dysfunction and vascular remodelling in PAH. To address this, we investigated (a) the expression of CLIC4 in the plasma of idiopathic PAH (IPAH) patients and 3 experimental models of PAH, (b) the effect of CLIC4 knockout on pulmonary vascular homeostasis in the hypoxic mouse, (c) the role of CLIC4 in the regulation of endothelial junctional integrity and function in human pulmonary artery endothelial cells and endothelial cells derived from...
circulating progenitors in patients with IPAH and (d) the molecular mechanisms mediating CLIC4-induced effects.

Materials and Methods
An expanded Materials and Methods section is available in the Online Data Supplement.

Human pulmonary arterial endothelial cell (HPAEC) and smooth muscle cell (HPASMC) culture

HPAECs were cultured in endothelial growth medium-2 (Promocell) while HPASMCs were cultured in smooth muscle cell growth medium-2 (Promocell). The cells were incubated under normoxic (20% O₂, 5% CO₂) or hypoxic (2% O₂, 5% CO₂, 93% N₂) conditions for 1-24 hr at 37°C. Hypoxia was used as means of manipulating HPAEC phenotype, protein expression and angiogenic potential in vitro. In some experiments, 5 μmol/L diphenylene iodonium (DPI, Sigma) or 5 μmol/L rotenone (Sigma), were added to the cells 1 hr prior to hypoxic exposure.

Plasma and blood-derived human endothelial cell from IPAH patients

Venous blood samples were obtained with local ethics committee approval and informed written consent from healthy volunteers and IPAH patients (Tables S1-S2 in the Online Data Supplement). Plasma samples were obtained from 123 consecutive patients, between 2011 and 2013, and endothelial colony forming cells (ECFCs) were derived as previously described¹¹,¹².

Animals and Experimental Design

All experiments were conducted in accordance to the UK Home Office Animals (Scientific Procedures) Act 1986 (London, UK) and the Colorado State University Animal Care Use Committee. To study the effect of CLIC4 gene knockout on the development of pulmonary hypertension, 6 weeks old wild-type or CLIC4 knockout adult male mice (CD-1 background)¹³,
were either housed in (1) normoxic conditions at sea level, (2) at Denver altitude (5280 ft; Pb=630 mmHg) for 13 weeks or (3) at Denver altitude for 10 weeks followed by 3 weeks hypoxia (normobaric; Fio₂=10%) (n=4–6/group). At 19 weeks, animals were weighed and anesthetized (Hypnorm 0.25 mL/kg; Midazolam 25 mg/kg IP), then the right ventricular systolic pressure (RVSP) was measured and the right ventricle to the left ventricle plus septum ratios (RV/LV+S) were determined. The left lungs were fixed, embedded in paraffin, and stained by the elastic van Gieson’s method. Vascular muscularisation was defined as the proportion of vessels (<50 μm diameter) with double elastic lamina over the total number of vessels stained with elastin. In addition, CLIC4 expression was studied in the lungs of rats with chronic hypoxia-, monocrotaline- and Sugen 5416/hypoxia models of pulmonary hypertension. Sugen 5416/hypoxia lung sections were a kind gift of Professor Nobert Voelkel (Virginia Commonwealth University, Richmond, VA, USA).

**CLIC4 protein expression and localization**

Plasma CLIC4 levels were measured using a sandwich enzyme immunoassay (Antibodies Online Inc., Aachen, Germany), according to the manufacturer’s protocol. Tissue CLIC4 protein expression and localization was investigated by western blot analysis, immunostaining of tissue sections and cultured endothelial cells followed by confocal microscopy. Relative quantification of CLIC4 expression in the intimal and medial layers of pulmonary vessels from healthy and pulmonary hypertension animal models was performed using Image J.

**Manipulation of CLIC4 expression and activity in cultured cells**

Overexpression CLIC4 was induced by adenoviral gene transfer. The inhibition of CLIC4 expression was achieved using adenoviral gene transfer of CLIC4 shRNA.

**Endothelial cell permeability and morphology**
Endothelial permeability and morphology were studied as previously described\textsuperscript{16}. In some experiments, mouse monoclonal VEGF function-blocking antibodies (10 µg/mL; R&D Systems) were added to the upper chamber of Transwell dishes 24 hr before permeability measurement.

\textit{In vitro angiogenesis assays}

Matrigel tube formation assay was used. Total tube length was determined using Image J software.

\textbf{Cell metabolic activity}

CellTiter 96 proliferation assay (Promega) was used to assess metabolic activity associated with cell proliferation and migration\textsuperscript{17}.

\textbf{Cell survival assay}

HPAECs were grown in full medium or medium deprived of serum and growth factors. Menadione (50 µM, Sigma) was added to cells for 6 hr as a positive control. Cells were incubated with MitoProbe\textsuperscript{TM} DiOC\textsubscript{2}(3) (Life Technologies, Invitrogen, USA) for 45 min and images captured using an Olympus IX70 inverted fluorescent microscope, with 10x objective F-view Soft Imaging System camera. Fluorescence intensity, corresponding to the number of live cells was measured with Image J.

\textbf{Short interference (si)RNA knockdown}

Cells were transfected with HIF-1α siRNA or BMPR2 siRNA or scrambled siRNA, and the levels of either CLIC4 or HIF-1α were measured by western blotting and/or by quantitative reverse transcription polymerase chain reaction 48 hr post-transfection.

\textbf{HIF-1α stabilization, endothelin-1 (ET-1) and VEGF production}

Human osteosarcoma cells stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE-luc)\textsuperscript{18} were used to assess the effects of CLIC4 on HIF
stabilization. Expression of HIF proteins was determined by western blotting and inhibition of HIF-1α expression was achieved by transfecting HPAECs with siRNA (details in the Online Data Supplement). Production of VEGF and other angiogenic factors was studied using Proteome Profiler Human Angiogenesis Array Kit® (R&D Systems) in untreated and CLIC4 overexpressing cells cultured under both normoxic and hypoxic conditions for 24 hr. Potential modulation of VEGF receptor type 2 (VEGFR2) expression was measured using a PathScan® Total VEGFR-2 Sandwich ELISA kit (Cell Signaling). Levels of ET-1 were determined using Human Endothelin-1 QuantiGLO® ELISA kit (R&D Systems).

**p65 expression, phosphorylation and NFκB activity**

p65 expression and phosphorylation were analysed by western blotting. The antibodies were from Cell Signaling Technology, Denver USA. Adenoviral NFκB luciferase reporter construct (AdNFκB-Luc) and Luciferase Assay System (Promega) were used to measure NFκB activity. In some experiments, the NFκB inhibitor, BAY 117085 (10 μM/L; Sigma) was added to the U2OS-HRE-luc cells 24 hr before luciferase assay.

**Statistical analysis**

All experiments were performed at least in triplicate. Data are presented as the mean±standard error of mean. Comparisons between 2 groups were made using Student’s t-test (except for analysis of plasma CLIC4 levels which was by the Mann-Whitney U-test) while 3 or more groups were compared with ANOVA and Tukey’s post-hoc test using GraphPad Prism version 5.0. All key findings were confirmed by non-parametric statistical analyses (Mann-Whitney U-test or Kruskal-Wallis test as appropriate). Statistical significance was accepted for P<0.05.
Results

**Increased CLIC4 levels in plasma and cells from IPAH patients**

Following on from our previous finding of increased CLIC4 protein expression in lung tissues from PAH patients\(^5\), we measured circulating CLIC4 and found that plasma CLIC4 levels were significantly higher in IPAH patients (n=123) compared with healthy volunteers (n=64) (Figure 1A). Levels were elevated across all four WHO functional classes, independent of clinical measures of disease severity.

There is interest in the role of circulating endothelial progenitor cells in the pathogenesis of pulmonary vascular disease\(^6,20,21\). Cells derived from the peripheral blood of IPAH patients and healthy volunteers exhibited similar immunostaining for the endothelial markers, CD31, VE-cadherin and von Willebrand factor and were indistinguishable from mature endothelial cells (Figure S1 in the Online Data Supplement). Flow cytometry also demonstrated that these cells were positive for the endothelial markers CD31 (99.4±0.1%, n=16) and VEGFR-2 (87.1±1.8%, n=16), but negative (<0.1%) for the haematopoietic/monocytic marker CD45 and CD133.

Consistent with the findings in lung tissues and plasma samples, CLIC4 protein levels were also significantly elevated in blood-derived endothelial cells from IPAH patients (Figure 1B).

**Increased CLIC4 expression in the lungs of pulmonary hypertensive rats**

CLIC4 protein was examined in the lungs of rats exposed to monocrotaline, chronic hypoxia and hypoxia-combined with Sugen. To understand better the relationship of CLIC4 to pulmonary hypertension, lung CLIC4 levels were measured during the development of pulmonary hypertension in monocrotaline- and hypoxia-exposed rats. An early increase was detected in both models, before pulmonary hypertension is established (typically 3-4 weeks in the monocrotaline rat\(^22,23\) and 2 weeks in the hypoxic rat\(^24\) (Figure 1C-D). Further studies in the hypoxic rat...
showed that this response appears to be lung-specific, as renal and cardiac CLIC4 levels were reduced in hypoxic rats (Figure S2 in the Online Data Supplement). CLIC4 was localized predominantly to the endothelium in the rat pulmonary vasculature (Figure 1E). Sections of lung from all 3 rat models of pulmonary hypertension exhibited characteristic medial wall thickening, distal muscularization and occlusion of pulmonary arteries, with prominent CLIC4 immunostaining of the endothelium, showing a similar distribution to the endothelial marker, von Willebrand factor (Figure 1E ii-vi). Cells in the hypertrophied medial layer of pulmonary arteries displayed weaker and more variable CLIC4 immunostaining (Figure 1E iii, v). Semi-quantitative analysis of immunostained lung sections indicated significantly increased CLIC4 expression in the intimal layer of intrapulmonary arteries in all three animal models of pulmonary hypertension, while the lower levels of CLIC4 immunostaining in the medial layer only increased significantly in the monocrotaline model (Figure S3 in the Online Data Supplement).

**CLIC4 knockout mice are protected against development of chronic hypoxia-induced pulmonary hypertension**

To determine the importance of CLIC4 signalling *in vivo*, we studied the development of chronic hypoxia-induced pulmonary hypertension in CLIC4 knockout (CLIC4 KO) mice, compared with wildtype (WT) controls. The absence of CLIC4 in CLIC4 KO mice was confirmed by western blotting and immunohistochemical analyses (Figure S4A-B in the Online Data Supplement). Knockout of CLIC4 protein did not alter the expression of the closely related CLIC1 or CLIC5 proteins (Figure S4A in the Online Data Supplement). WT mice kept for 13 weeks at Denver altitude (5280 feet above sea level) developed pulmonary hypertension and vascular remodelling, evident by a significant increase in RVSP, RV/LV+S ratio and vessel muscularization (Figure
2). In contrast, these measurements showed little change in CLIC4 knockout mice maintained under identical conditions. Exposure of these mice to an additional 3 weeks of hypoxia (10% O₂) further increased vessel muscularisation and RV hypertrophy while RVSP remained unchanged. The effects of hypoxia on vessel muscularization, right heart hypertrophy and RSVP were significantly attenuated in CLIC4 KO mice (Figure 2). These results suggest that CLIC4 knockdown is protective in early stages of pulmonary hypertension.

**CLIC4 affects HPAEC morphology in vitro**

The intracellular localization of CLIC4 is an important determinant of its function\(^\text{15}\). Soluble, cytoplasmic CLIC4 can localize to the cell membrane where it interacts with cytoskeletal proteins involved in lamellipodia and filopodia formation, while nuclear-targeted CLIC4 promotes apoptosis\(^\text{15}\). CLIC4 was localised predominantly in the cytoplasm of cultured HPAECs (Figure S5 in the Online Data Supplement), consistent with its distribution in cells in plexiform lesions\(^\text{5}\). Hypoxia increased CLIC4 protein expression 2-fold, with some translocation of protein to the cell periphery (Figure S5A, B in the Online Data Supplement). Hypoxia-induced increase in CLIC4 expression in endothelial cells was prevented by the inhibitors of intracellular ROS production, DPI and rotenone (Figure S5B in the Online Data Supplement). In contrast, hypoxia did not significantly affect CLIC4 expression in HPASMCs (Figure S6 in the Online Data Supplement). We have also compared the relative levels of CLIC4 protein expression in HPAECs and HPASMCs cultured in normoxic or in hypoxic conditions. The results show that normoxic and hypoxic levels of CLIC4 expression in HPAECs were significantly higher than CLIC4 levels in HPASMCs (Figure S6 in the Online Data Supplement).

To examine the role of CLIC4 in modulating pulmonary endothelial cell phenotype, CLIC4 was overexpressed in HPAECs via adenoviral gene transfer. The CLIC4-overexpressing
cells showed enhanced cortical F-actin polymerization and the formation of membrane ruffles, absent in the normal, untreated endothelium (Figure S7A-D in the Online Data Supplement).

The morphological effects of CLIC4 overexpression were inhibited in cells co-transfected with CLIC4 shRNA (Figure S7E-F in the Online Data Supplement).

**CLIC4 affects hypoxia-induced endothelial permeability**

We hypothesised that CLIC4, through association with cytoskeletal proteins and regulation of cell morphology could influence vascular integrity. Increased endothelial permeability is thought to be an early event in the pathogenesis of pulmonary hypertension, permitting growth factors access to cells in the vascular wall. Hypoxia affected both HPAEC cell morphology and permeability (Figure 3A, B). In confluent cells under normal oxygen tension, F-actin was localized mainly at the cell periphery together with vascular endothelial (VE)-cadherin (Figure 3A), a major component of adherence junctions. Changes in CLIC4 protein levels following adenoviral infection were confirmed by western blotting (Figure 3B). Hypoxia (24 hr) induced the formation of actomyosin filaments and disruption of intercellular junctions and these changes were accompanied by a 1.7-fold increase in endothelial permeability (Figures 3A, C).

Overexpression of CLIC4 compromised endothelial barrier function under normoxic conditions and further increased hypoxia-induced permeability (Figure 3C). Conversely, the effects of hypoxia were inhibited by CLIC4 shRNA (Figures 3A, C).

**CLIC4 expression influences HPAEC metabolic activity, cell survival and angiogenesis in vitro**

The reduction of MTS tertazolium compound into coloured formazan measures the metabolic activity of viable cells, which increases with cell proliferation and migration, irrespective of the presence of oxidative respiration. Overexpression of CLIC4 increased cell metabolic activity in
both normoxic and hypoxic conditions (Figure 4A). Endogenous CLIC4 expression was essential for hypoxia-induced cell activation and was inhibited by treatment with CLIC4 shRNA (Figure 4A). CLIC4 overexpression significantly improved survival of HPAECs cultured in media deprived of serum and growth factors (Figure 4B), indicating its possible role in the emergence of apoptosis-resistant endothelial cells in the distal pulmonary vasculature.

Hypoxia, a known stimulus of angiogenesis, induced a 3-fold increase in tube formation by HPAECs in Matrigel, compared with normoxic controls. Overexpression of CLIC4 increased tube formation in both normoxic (2-fold) and hypoxic (5.8-fold) conditions compared to controls. The effects of hypoxia and CLIC4 overexpression were prevented by CLIC4 shRNA (Figure 4C, D).

CLIC4 expression is not affected by BMPR2 knockdown

Reduction in BMPR2 signaling is associated with the pathogenesis of PAH. We used BMPR2 siRNA to inhibit expression of the receptor in both HPAECs and blood-derived endothelial cells but found no change in CLIC4 protein levels in either cell type, despite a marked reduction in BMPR2 mRNA and protein levels (Figure S8 in the Online Data Supplement).

CLIC4 stabilises HIF-1α and increases production of ET-1 and VEGF in HPAECs

Increased HIF-1α stabilization and activity has been reported in the pulmonary vasculature and endothelial cells isolated from patients with IPAH. Knockout of the murine CLIC4 is associated with a reduction of ischemia-induced HIF-1α signalling. We postulated that the effects of CLIC4 in HPAECs may reflect, at least in part, modulation of HIF-1α activity and expression. To measure changes in HIF activation, we used the human U2OS-HRE-luc cell line, which had been optimised and validated for the use in high-throughput screens of HIF inhibitors and studies on hypoxia-induced HIF activation. Overexpression of CLIC4 led to a marked (30-
fold) increase in U2OS luciferase activity under hypoxic conditions, which was reduced to adenovirus control levels by pre-treatment with CLIC4 shRNA (Figure 5A). In keeping with a role for CLIC4 in the regulation of HIF stabilization, overexpression of CLIC4 was accompanied by the nuclear translocation of HIF-1α in HPAECs (Figure 5B) and a marked increase of HIF-1α protein levels (Figure 5C). Consistent with the changes in HIF-1 activity, CLIC4 increased production of ET-1 in cells, while CLIC4 shRNA had an inhibitory effect (Figure 6A). The overexpression of CLIC4 was also associated with increased VEGF production in HPAECs (Figure 6B and Figure S9A in the Online Data Supplement), with no apparent change in VEGFR-2 levels (Figure S9B in the Online Data Supplement). VEGF function-blocking antibodies prevented the CLIC4-induced increase in endothelial permeability, implicating secreted VEGF as a critical mediator of CLIC4-induced changes in endothelial junctional integrity (Figure S9C). siRNA-mediated knockdown of HIF-1α (Figure S10A in the Online Data Supplement) completely prevented the pro-angiogenic effects of CLIC4 (Figure 6C), reinforcing the concept of HIF-1α acting as a downstream mediator of CLIC4 effects. Consistent with the postulated role for CLIC4 in the regulation of HIF stability, CLIC4 knockout mice showed reduced levels of HIF-1α (Figure S10B in the Online Data Supplement). Interestingly, the levels of HIF-2α in these mice were also reduced, suggesting common regulatory mechanisms (Figure S10B in the Online Data Supplement).

HIF-1α and tube formation are dysregulated in blood-derived endothelial cells from IPAH patients

Cells derived from circulating progenitor cells in IPAH patients showed significantly higher levels of CLIC4 and HIF-1α proteins, compared with cells isolated from healthy volunteers (Figure 7A-B). In keeping with the proposed pro-angiogenic role of CLIC4, endothelial cells
from IPAH patients also showed an enhanced capacity to form endothelial tubes in Matrigel. Transfection with CLIC4 shRNA reduced CLIC4 expression and tube formation to a level comparable to that seen with cells derived from healthy volunteers (Figures 7C).

**CLIC4-induced activation of NFκB is required for HIF activation**

The pro-inflammatory transcription factor NFκB is a known inducer of HIF activity\(^{27}\). Using affinity purification followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) analyses (data not shown), the approach employed to identify CLIC4 in human lung\(^5\), we identified a p65 subunit of NFκB (RelA) as one of the proteins interacting with CLIC4. Phosphorylation of p65 facilitates nuclear translocation and transcriptional activity of NFκB\(^{28}\) and we hypothesised that CLIC4 overexpression may affect NFκB activity.

CLIC4 overexpression in HPAECs significantly increased NFκB activity in normoxic and in hypoxic conditions (Figure 8A). NFκB activation in CLIC4 overexpressing cells was accompanied by increased phosphorylation of p65 on Ser536 (Figure 8B), which is known to facilitate nuclear translocation and transcriptional activation of NFκB\(^{25}\). Consistent with the postulated role of CLIC4/NFκB in the regulation of HIF activity, treatment with an NFκB inhibitor, BAY-117085, attenuated CLIC4-induced HIF activation in hypoxic HPAECs (Figure 8C) – suggesting NFκB as an upstream regulator of HIF in the CLIC4 signalling pathway.

**Discussion**

This study demonstrates a critical role for CLIC4 in the regulation of pulmonary endothelial function and vascular homeostasis. Increased CLIC4 levels were observed in plasma and blood-derived endothelial cells from IPAH patients and in the vascular endothelium of three rodent models of pulmonary hypertension. CLIC4 gene knockout in mice attenuated the development of
chronic hypoxia-induced pulmonary hypertension. Overexpression of CLIC4 in HPAECs increased endothelial permeability and influenced their survival and angiogenic capacity, these effects being dependent in part on the stabilisation of HIF-1α. Selective inhibition of CLIC4 expression in endothelial cells derived from circulating progenitors from IPAH patients inhibited the aberrant tube formation characteristic of these cells. The mechanism of CLIC4-induced effects involves p65-dependent NFκB activation followed by stabilisation of HIF. Coupled with our previous observation of increased CLIC4 expression in the remodelled pulmonary vasculature of PAH patients⁵, our data provide compelling evidence implicating CLIC4 in the pathobiology of the disease.

As in IPAH lungs, increased CLIC4 expression was observed most consistently in the endothelium of the pulmonary vasculature of all 3 rat models of pulmonary hypertension - chronic exposure to hypoxia, monocrotaline and the combination of Sugen 5416 and hypoxia, a model that exhibits similarities to the pathological features of advanced human IPAH¹⁴. Nonetheless, while the endothelium appears to be a predominant site of CLIC4 protein expression in the hypertensive human and animal lung, we cannot exclude the possibility that CLIC4 may also affect other cell types. We observed lower levels of CLIC4 immunostaining in the pulmonary vascular medial layer, but this was increased in the vessels of monocrotaline-treated rats and CLIC4 has been implicated in the migratory responses of cultured HPASMCs downstream of BMPRII/BMP-2 signaling¹⁰.

The endothelial layer of the pulmonary vasculature acts as a barrier to the underlying tissue, limiting the influx of inflammatory cells, growth factors and cytokines. Changes in vascular endothelial permeability are thought to occur early in the pathogenesis of pulmonary hypertension, both in animal models and human PAH²⁹,³⁰. Consistent with a role for CLIC4 in
compromising endothelial integrity early in the disease, we found that CLIC4 expression is increased (by 7 days) in both the monocrotaline and hypoxia-induced rat models of pulmonary hypertension, before pulmonary hypertension and vascular remodelling is fully established in these models (3-4 weeks for the monocrotaline rat and around 2 weeks for the hypoxic rat).

In contrast to the lung, CLIC4 levels were reduced in renal and myocardial tissues of chronically hypoxic animals. This suggests an organ-specific response to hypoxia and the differential regulation of CLIC4 expression in pulmonary and systemic vascular beds. The potential drivers of increased CLIC4 expression in the lung in pulmonary hypertension include oxidative stress, growth factors and pro-inflammatory cytokines (e.g. TNF-α and TGF-β) \(^4,8,26,31-33\). Hypoxia-response element consensus sequences have also been identified in the CLIC4 gene and may be a regulatory mechanism contributing to increased CLIC4 expression in ischemia or hypoxic conditions \(^4\). Our preliminary observations suggest that ROS generation may play a role in the regulation of CLIC4 expression in pulmonary endothelium, consistent with observations in ovarian cancer \(^8\). The inhibitor of NADPH-oxidase-induced ROS formation, DPI, and the inhibitor of mitochondrial ROS, rotenone, both attenuated the hypoxia-induced increase in CLIC4 expression in HPAECs.

As heterozygous germline BMPR2 mutations contribute to heritable forms of PAH \(^34\) and down regulation of BMP signalling has been implicated in the development of experimental pulmonary hypertension \(^35\), we examined whether BMPR2 expression influenced CLIC4 protein levels. Despite a marked suppression of BMPR2 mRNA and protein expression, we found no significant change in CLIC4 protein levels in either HPAECs or blood-derived endothelial cells. This is consistent with the results of an earlier study on the regulation of CLIC4 mRNA expression in isolated smooth muscle cells \(^10\) and suggests that the enhanced expression of CLIC4
found in endothelial cells from IPAH patients and lungs from rats with pulmonary hypertension is not a direct manifestation of altered BMPR2 expression.

In addition to increasing permeability, CLIC4-induced endothelial junctional remodelling is an important step in angiogenesis, as breaking off inter-endothelial junctions in activated endothelium is a prerequisite for endothelial cell migration and new vessel formation. Key mediators of the effects of increased CLIC4 expression are HIF and angiogenic factors, such as VEGF. Hypoxia-inducible transcription factors have been implicated in both the development of experimental pulmonary hypertension and the altered endothelial cell phenotype found in IPAH patients. HIF-1α regulates release of pro-angiogenic factors such as VEGF and vasoactive mediators such as ET-1, both of which are elevated in PAH. HIF-1α has also been reported to mediate the effects of hypoxia on endothelial permeability. We found that CLIC4 overexpression in HPAECs led to HIF activation in normoxia and to a marked increase in HIF activity under hypoxic conditions. This was accompanied by nuclear translocation of HIF-1α and increased VEGF and ET-1 production, while VEGF inhibition reduced the barrier-compromising effects of CLIC4. Consistent with these observations, CLIC4 knockout mice show impaired ischemia-induced expression of HIF-1α, VEGF and angiopoietin-2. We cannot exclude the possibility that HIF-2 may also play a role. HIF-2 is induced by hypoxia and regulates endothelial cell proliferation and angiogenesis and we observed a reduction in HIF-2 expression in CLIC4 knockout mice, contemporaneous with the reduction in HIF-1 expression.

Finally, we identified the p65 subunit of the pro-inflammatory transcription factor NFκB as one of the CLIC4-interacting proteins in endothelial cells. Phosphorylation of p65 on serine residues S536 and Ser276 is critical for full activation of NFκB. CLIC4 competes with phosphatase PPM1A, known to de-phosphorylate p65 and other signalling proteins, including
Smad 2/3\textsuperscript{41}. We observed significant activation of NFκB following overexpression of CLIC4, which correlated with increased phosphorylation of p65 on Ser536. More importantly, the NFκB inhibitor BAY117085, known to inhibit nuclear translocation of p65 in endothelial cells\textsuperscript{42}, significantly attenuated CLIC4-induced activation of HIF. It is well documented that NFκB regulates HIF activity in a variety of pathological states\textsuperscript{27}. These findings suggest NFκB acts as an upstream activator of HIF in the CLIC4 signalling pathway. A proposed CLIC4 signalling pathway is shown in Figure 8D.

**Conclusion**

Pulmonary endothelial CLIC4 is up-regulated in human PAH and experimental models of pulmonary hypertension. CLIC4 regulates the activity of two key transcription factors, NFκB and HIF-1, that drive endothelial responses to inflammatory and angiogenic stimuli. CLIC4 inhibition serves to maintain pulmonary endothelial function under hypoxic stress and attenuates the development of pulmonary hypertension in the chronically hypoxic mouse. Approaches that reverse increased pulmonary endothelial CLIC4 expression may be beneficial in patients with PAH.

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Conflict of Interest Disclosures: None.

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**Figure Legends:**

**Figure 1.** CLIC4 protein expression in patient plasma, human ECFCs and rat lung. (A) CLIC4 protein levels in plasma samples from IPAH patients (n=123) and healthy volunteers (n=64). As these data show a skewed distribution they were analysed using the Mann-Whitney U-test. (B) Levels determined in blood-derived endothelial cells from IPAH patients (n=4-5). (C) CLIC4 protein expression in the lungs of rats treated with monocrotaline (MCT) or (D) exposed to hypoxia (n= 3-8). Data are presented as mean±SEM. *P<0.05; **P<0.01, compared with
controls. Lung sections from (E i) control, (E ii) hypoxia-, (E iii-iv) MCT and (E v-vi) Sugen 5416/hypoxia-induced pulmonary hypertensive rats, demonstrating co-localisation of CLIC4 and von Willebrand factor (vWF) in the pulmonary endothelium (arrows) of normal and remodelled pulmonary arteries. Arrowheads (E iii, v) indicate CLIC4 immunoreactivity in smooth muscle cells. Bar=50 μm.

**Figure 2.** CLIC4 gene knockout attenuates development of chronic hypoxia-induced pulmonary hypertension. (A) Percentage of muscularized vessels diameter ≤50 μm in lung sections of normoxic and hypoxic wildtype (WT; black bars) and CLIC4 knockout (CLIC4 KO; grey bars) mice. (B) Representative sections showing α-smooth muscle actin staining in normoxic or hypoxic WT or CLIC4 KO mice. Red arrows point to the fully muscularized peripheral artery with double elastic lamina staining. Bar=50 μm. (C) Right ventricular hypertrophy (RV/(LV+S)) and (D) right ventricular systolic pressure (RVSP) were measured in the WT control and CLIC4 KO mice following exposure to normoxia, 13 weeks at Denver altitude or 10 weeks (w) of Denver altitude followed by 3 weeks of hypoxia. *P<0.05, **P<0.01, ***P<0.01, compared to normoxic WT; #P<0.05, ##P<0.01 compared to WT at Denver altitude and @@P<0.01, WT versus CLIC4 KO group at 3w Hypoxia+10w Denver Altitude; (C). In (A-C) n=4-8.

**Figure 3.** Hypoxia-induced changes in human pulmonary endothelial phenotype and permeability. (A) Images showing the organization of F-actin and VE-cadherin in confluent HPAECs under normoxic or hypoxic (24 hr) conditions. The cells were left untreated or overexpressed CLIC4 shRNA, as indicated. Merged images show F-actin (red), VE-cadherin (blue) and GFP co-expressed with CLIC4 shRNA (green). Bar=10 μm. (B) The adenoviral
manipulation of CLIC4 expression was confirmed by western blot analysis of cell extracts. (C) Pulmonary endothelial cell permeability in normoxic and hypoxic cells expressing scrambled shRNA, CLIC4 shRNA or CLIC4, as indicated; In (C) data are expressed as % of control and each bar represents mean±SEM (n=4). *P<0.05; ***P<0.001, compared with normoxic AdControl; ###P<0.001, compared with hypoxic AdControl. Comparisons between AdCLIC4 and CLIC4 shRNA + AdCLIC4 are also indicated.

**Figure 4.** Effects of CLIC4 expression on HPAEC metabolic activity, cell survival and angiogenesis. (A) Cell metabolic activity under normoxic and hypoxic conditions and following overexpression of CLIC4, scrambled shRNA (shRNA control) or CLIC4 shRNA, as indicated; MTS reduction assay. (B) HPAEC viability in cells cultured in full media, treated with apoptosis-inducer, menadione (6 hr, 10 μM) or serum-starved for 48 hr. The cells were overexpressing CLIC4 or CLIC4 and CLIC4 shRNA, as indicated. (C) Changes in total endothelial tube length (expressed as % of normoxic control) in cells treated, as indicated. (D) Representative images showing the effects AdCLIC4 and AdCLIC4 shRNA on tube formation in normoxic and hypoxic HPAECs. Bar=500 μm. In (A-C) each bar represents mean±SEM; (n=4-5). *P<0.05, **P<0.01, ***P<0.001, compared with normoxic control (A-C) or non-starved control (B); ###P<0.001, compared with hypoxic AdControl (A, C). Comparisons between AdCLIC4 and AdCLIC4 + CLIC4 shRNA groups are also indicated.

**Figure 5.** CLIC4 regulates endothelial HIF activity and stabilization. Overexpression of CLIC4 (24 hr) or exposure to hypoxia (A) stabilizes HIF in U2OS-HRE-luc cells, luciferase reporter assay (n=12); (B) increases nuclear translocation of HIF-1α and (C) increases HIF-1α levels in
HPAECs (n=4-5). Each bar represents mean±SEM ***P<0.001, **P<0.01, compared with normoxic AdControl; ###P<0.001, compared with hypoxic AdControl. Comparisons between AdCLIC4 and CLIC4 shRNA + AdCLIC4, are also indicated

**Figure 6.** The effect of CLIC4 on HIF-dependent protein expression and tube formation in HPAECs. Production of (A) ET-1 and (B) VEGF in normoxic and hypoxic HPAECs overexpressing CLIC4 or CLIC4 shRNA (n=3-5). (C) HPAEC tube formation following siRNA-mediated HIF-1α knockdown in cells treated, as indicated (n=5). Each bar represents mean±SEM. *P<0.05; **P<0.01, ***P<0.001 compared with normoxic AdControl; ###P<0.01, ###P<0.001 compared with hypoxic AdControl. Comparisons between AdCLIC4 and CLIC4 shRNA or between scrambled siRNA and HIF-1α siRNA groups are also indicated.

**Figure 7.** HIF-1α levels and tube formation in blood-derived endothelial cells. (A) Blood-derived endothelial cells from IPAH patients showed greater protein expression of HIF-1α compared to healthy controls. (B) CLIC4 shRNA (CSh; 48h) reduces CLIC4 levels in IPAH cells to control levels, while scrambled shRNA (Ssh) has no effect. (C) CLIC4 shRNA inhibits tube formation in IPAH endothelial cells. Each bar represents mean±SEM (n=3-4). Representative images of tube formation in healthy and IPAH cells are shown below the graph; Bar=100 μm. In (A-D) *P<0.05, **P<0.01, ***P<0.001, compared with untreated cells derived from healthy volunteers. #P<0.05, ###P<0.01 comparison with untreated IPAH cells.

**Figure 8.** CLIC4 regulates NFκB activity in HPAECs. (A) NFκB activity in HPAECs treated with lipopolysaccharide (LPS), control adenovirus (AdControl), AdCLIC4 or AdCLIC4 shRNA
in normoxic (grey bars) or hypoxic (black bars) conditions, as indicated; luciferase reporter assay. (B) CLIC4 increases phosphorylation of p65 on Serine 536, western blotting. (C) NFκB inhibitor, BAY 117085 prevents HIF activation in CLIC4- overexpressing HPAECs (30 hr overexpression) in hypoxia. n=4; *P<0.05; **P<0.01, ***P<0.001, compared with untreated controls; #P<0.05, ##P<0.01, ###P<0.001, comparison with hypoxic controls. (D) The proposed CLIC4 signalling pathway.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
**Figure 8**
Aberrant Chloride Intracellular Channel 4 Expression Contributes to Endothelial Dysfunction in Pulmonary Arterial Hypertension


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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

**Human pulmonary artery endothelial and smooth muscle cell culture**

Primary human pulmonary artery endothelial cells (HPAECs) were obtained from PromoCell (Heidelberg, Germany) and cultured in endothelial cell growth medium-2 (PromoCell). The cells were grown on fibronectin-covered (bovine fibronectin, Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK) plastic ware and cultured under normal oxygen tension (20% O\(_2\), 5% CO\(_2\)) or exposed to hypoxia (2% O\(_2\), 5% CO\(_2\), 93% N\(_2\)) for 1-24 h. In some experiments, ROS inhibitors, 5 µmol/L diphenylene iodonium (DPI, Sigma) or 5 µmol/L rotenone (Sigma), were added to the cells 1 hr prior to hypoxic exposure. Human pulmonary artery smooth muscle cells (HPASMCs) were cultured in smooth muscle cell growth medium-2 (Promocell).

**Blood-derived human endothelial cell isolation and characterization**

Human endothelial cells, also known as endothelial colony forming cells (ECFCs) or late outgrowth endothelial progenitor cells, were derived from peripheral blood\(^{31,2}\). Venous blood samples (~50 ml) were collected in EDTA vacutainers, diluted 1:1 with phosphate buffered saline (PBS) containing 0.2% EDTA and 2% foetal bovine serum (FBS) and layered onto Ficoll Plaque PLUS (GE Healthcare, Amersham, UK) for density gradient centrifugation. Peripheral blood mononuclear cells (MNCs) were aspirated, washed in PBS and re-suspended in EGM-2 medium (CC-3156, Lonza Biologics, Slough, UK), supplemented with growth factors (CC-4176, EGM™-2 bullet kit, Lonza), 20% FBS (HyClone, Thermo Scientific, South
Logan, UT, USA) and 1% antibiotic/antimycotic solution (Gibco, Invitrogen, Paisley, UK). Cells were seeded (3 to $5 \times 10^7$ MNCs in 4 ml of medium per well) in 6 well plates coated with type 1 rat tail collagen (BD Biosciences, Bedford, MA, USA). The medium was replaced daily for the first week (removing non-adherent cells), and every 2 days thereafter. Individual colonies were harvested by attaching a cloning cylinder (8 mm or 10 mm in diameter, Millipore, Watford, UK) with vacuum grease and using warm 0.05% Trypsin-EDTA solution (Invitrogen) to remove all the cells within the cylinder. Dislodged cells were re-suspended in fresh EGM-2 medium, seeded (3000 to 5000 cells/cm$^2$) in 1% gelatin pre-coated culture vessels and propagated when 70-80% confluent. Distinct colonies of cells, capable of serial propagation for at least 8 serial passages and clonal growth, and displaying a stable population doubling time as well as endothelial phenotype, were used between passages 4 to 7.

The endothelial lineage was confirmed by flow cytometry and immunostaining of cells grown on sterile glass coverslips (VWR International, Leicestershire, UK), prewashed with hydrochloric acid and coated with 1% gelatin. For lectin binding, live cells were incubated with FITC-conjugated *Ulex europaeus* agglutinin-1 (UEA-1) lectin (1:30, L9006, Sigma-Aldrich) in EGM-2 medium for 1hr in a humidified incubator (5% CO$_2$, 37°C). The coverslips were then washed in Tris-buffered saline (TBS) before being fixed in 4% w/v paraformaldehyde (PFA) in PBS for 20 min at 4°C. For immunostaining, the cells were first fixed in 4% PFA in PBS (w/v) for 20 min at 4°C, washed twice in PBS and immersed in 3% bovine serum albumin in TBS for 30 min at room temperature. Cells were then incubated overnight at 4°C with either mouse monoclonal anti-human CD31; (JC/70A, Dako, Glostrup, Denmark), anti-human VE-Cadherin (sc-9989, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-human von Willebrand factor (A0082, Dako). After washing, the
coverslips were incubated for 1 hour at room temperature with Alexa-488 green-conjugated secondary antibodies (Dako). The coverslips were washed again with TBS before being mounted in DAPI-containing Vectashield (Vector Laboratories Ltd, Peterborough, UK) and observed using fluorescence microscopy.

A three colour FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, Oxford, UK) was used for flow cytometry. Cells were suspended in Fc receptor blocker (Miltenyl Biotec, Bergisch Gladbach, Germany) for 5 min before being re-suspended for 30 min at 4°C with mouse IgG1 anti-human CD31 (555446, phycoerythrin (PE)-labelled, BD Pharmingen, Cowley, UK); VEGFR2/KDR (FAB357A, allophycocyanin (APC)-labelled, R&D Systems, Minneapolis, MN, USA); CD45 (345785, PE-labelled, BD Pharmingen); or CD133 (130-080-801, PE-labelled, Miltenyi Biotec). Fluorochrome matched isotype controls were used at the same dilution factor, as per the manufacturer’s recommendation (BD Pharmingen). The data was analysed using FCS Express software (De Novo Software, Los Angeles, CA, USA) and presented as the number of antigen expressing (positive) cells expressed as a proportion of the gated ECFC population.

Animal experiments.
All experiments were conducted in accordance to the UK Home Office Animals (Scientific Procedures) Act 1986 (London, UK) and the Colorado State University Animal Care Use Committee.

To study the effect of CLIC4 gene knockout on the development of pulmonary hypertension, 6 weeks old wild-type or CLIC4 knockout adult male mice (CD-1 background, generated by Professor John C EdwardsS3), were either housed in (1) normoxic conditions at sea level, (2)
The expression of CLIC4 protein in tissues and cells was examined by western blotting and immunostaining. Proteins were separated by 10% NuPAGE® Bis-Tris gels (Invitrogen) using
MES buffer at 200V constant for 35 minutes (5 ug equivalent of protein per lane). Membranes were probed with a mouse monoclonal antibody raised against a synthetic peptide sequence (238-250 amino acids) of human CLIC4 (sc-135739, Santa Cruz Biotechnology; 0.2 µg/mL). Controls included the use of mouse monoclonal antibodies against human vinculin (ab18058, Abcam PLC, Cambridge, UK; 2 µg/ml) and β-Actin (A2228, Sigma: 1:10,000 dilution). The relative intensity of the immunoreactive bands was determined by densitometry using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

The localization of CLIC4 in tissue sections was determined by immunostaining. Dewaxed and rehydrated lung sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate (pH 6.0) and 0.05% Tween 20, at 80°C for 20 minutes, and immunostained using the avidin-biotin-peroxidase complex (ABC Elite, Vector Laboratories) method and 3,3’-diaminobenzidine as a substrate. Sections were incubated with a mouse monoclonal antibody raised against a synthetic peptide corresponding to amino acids 238-250 of human CLIC4 (sc-135739, Santa Cruz Biotechnology; 20 µg/ml) and a polyclonal rabbit antibody raised against human von Willebrand Factor was used as an endothelial marker (A082, Dako; 1:1000 dilution). Controls included the use of affinity purified non-immune rabbit IgG instead of the primary antibodies.

Relative quantification of CLIC4 expression in the intimal and medial layers of 5 pulmonary vessels of equal size selected from lung sections of healthy, chronic hypoxia-induced, monocrotaline and hypoxia-Sugen PH animals was performed using Image J.
**siRNA knockdown of BMPR2**

To achieve knockdown of gene expression, endothelial cells were seeded in 6-well plates (200,000 cells per well). The following day, siRNA duplexes (375 pmol per well) specific for ALK5 (SI00301903; Qiagen, Crawley, UK), BMPR2 (VHS41350; Invitrogen, Paisley, UK) or a scrambled sequence of similar GC content (12935-200; Invitrogen) were transfected in OPTI-MEM medium (Invitrogen) using Oligofectamine (12252-011; Invitrogen), according to the manufacturers guidelines for transfecting HUVECs. The efficiency of the knockdown was determined 48 and 72 hours after transfection by measuring protein and mRNA levels in cell extracts, using western blotting and RT-PCR respectively.

To determine BMPR2 protein expression, cells were washed three times with PBS and extracted in 9M Urea/2% CHAPS (Sigma-Aldrich, Dorset, UK). Protein concentration was determined by Bradford assay (Bio-Rad, Hertfordshire, UK) according to manufacturer instruction and equal amounts of denatured protein (50 µg per lane) were separated by SDS-PAGE (4 – 12% NuPAGE® Novex Bis-Tris Gel 1.5 mm, 10 well; Invitrogen) and transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, 0.2 µm pore size; GE Healthcare, Amersham, Buckinghamshire, UK). After blocking in TBS with 5% BSA for 1 h, membranes were incubated overnight at 4°C with a mouse monoclonal antibody (1:300 dilution) against human BMPR2 (612292; BD Biosciences, Oxford, UK) in TBS with 0.1% Tween (TBS-T) and 5% BSA. After washing 5x with TBS-T, membranes were incubated for 1 hr at room temperature with HRP-labelled sheep anti-mouse IgG at 1:2,000 dilution (NXA931; GE Healthcare), washed 5x with TBS-T and visualized by chemiluminescence (Luminata™ Forte Western HRP substrate; Millipore, Watford, UK). The relative intensity of the immunoreactive bands was determined by densitometry and normalized for cell protein by
reprobing the membrane with a mouse monoclonal antibody against human β-actin (1:2000 dilution; A1978; Sigma).

To assess mRNA levels, RNA was extracted using Trizol reagent (15596-026; Invitrogen) according to manufacturer instructions, digested with DNase I (AMPD1; Sigma) and cDNA reverse transcribed using the ThermoScript RT-PCR system (11146-016; Invitrogen). PCR reactions were prepared with 40 ng of cDNA in a 20 µl reaction volume with 10 µl of PCR MasterMix (M7502; Promega, Southampton, Hampshire, UK) and 0.5 µM of primers with sequences (annealing temperatures and cycle number) as follows: ALK5 (57.5°C, 34 cycles) sense: 5’-CGTGCTGACATCTATGCAAT-3’ and antisense: 5’-AGCTGCTCCATTGGCATA-3’; BMPR2 (53°C, 30 cycles) sense: 5’-CTGCGGCTGCTTCAGGAGGCTGCACA-3’ and antisense: 5’-TGTTGTTGTCAGGAGGCT-3’; CLIC4 (50°C, 27 cycles) sense: 5’-GCTAAGGCTGCACTGATGCCTGCGA-3’ and antisense: 5’-CAGTGCTCCATTGGCATA-3’; β2-microglobulin (64°C, 26 cycles) sense: 5’-GGGTTTCATCCATGCCAAGGCA-3’ and anti-sense: 5’-TGGTTCACACCGGCGAGGACATAC-3’ (all synthesised by Invitrogen). Amplified products were run on 1% agarose gels stained with GelRed Nucleic Acid Gel Stain at 1:20,000 dilution (BT41003; Biotium, Hayward, CA, USA).

Quantitative PCR (qPCR) was prepared with 20 ng of cDNA in a 10 µl reaction volume using 5 µl of Platinum SYBR Green qPCR SuperMix-UDG with 0.2 µl ROX reference dye (11733-046; Invitrogen) and 0.5 µM of primers with sequences as follows: BMPR2 sense: 5’-CAAATCTGTGAGCCCAACGAC-3’ and anti-sense 5’-GAGGAAGAATAATCTGGATAAGGACATAC-3’; peptidylprolyl isomerase A (PPIA) sense: 5’-CTGCACTGCAAGACTGTA-3’ and anti-sense: 5’-GCCATTCCTGGACCCAAA-
3’; α-tubulin sense: 5’-GCCAAGCGTGCTTTGTTC-3’ and anti-sense: 5’-CACACCAACCTCTCATAATCC-3’ (all synthesised by Invitrogen). CLIC4 primers for qPCR were purchased from Qiagen (QT02312569; Qiagen). qPCR was run in a 7500 Fast Real-Time PCR System (4351106; Applied Biosystem, Paisley, UK). The efficiency of each primer set was confirmed to be above 90%. The relative expression of target mRNAs was determined using the standard curve method and was normalised by factors calculated from PPIA and tubulin expression using the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/).

**Manipulation of CLIC4 expression and activity in cultured cells**

Overexpression of CLIC4 (AdCLIC4, predominantly cytoplasmic) was induced by adenoviral (Ad) gene transfer\(^5\). AdCLIC4 was heme agglutinin (HA)- and myc-tagged and expressed under tetracycline control, allowing precise control of expression and localization. Cells were infected with adenoviruses at a multiplicity of infection (MOI) of 100 and left for 24 hr prior to further treatment or analysis. Cells infected with AdGFP or AdCLIC4 without AdTet-off were treated as adenoviral controls (AdControls).

Inhibition of CLIC4 expression was achieved with adenoviral gene transfer of CLIC4 shRNA (pEQU6-Clic4-shRNA-GFP; 5’-GCCGTAATGTTGAACAGAATT-3’, Welgen, Inc., Worcester, MA). For gene silencing experiments, pEQU6-controlshRNA-GFP (5’-CAACAAAGATGAGAGGCACC-3’) was used as control and shRNA constructs were transfected 24 hr before CLIC4 adenoviral gene transfer of AdCLIC4 (i.e. total expression time 48 h). The effects of CLIC4 gene silencing and overexpression were verified by western blotting.
**Endothelial cell permeability and morphology**

The effect of CLIC4 expression and hypoxia on transendothelial permeability was studied in HPAECs grown in Transwell-Clear filters (3-µm pore size, 12-mm diameter; Costar Corning, High Wycombe, UK). Changes in permeability were assessed by measuring the passage of fluorescent dextran (FITC-dextran, MW 40 kDa, Sigma) through the endothelial cell layer, as previously described\textsuperscript{66}. Cells were grown to confluency prior to adenoviral infection. 2 hr post-infection the cells were placed in normoxic or in hypoxic conditions and endothelial permeability was examined 24 hr later. In some experiments, mouse monoclonal VEGF function-blocking antibodies (10µg/ml; R&D Systems) were added to the upper chamber of Transwell dishes 24 hr before permeability measurement. Mouse monoclonal anti-HA antibody (sc-805, Santa Cruz Biotechnology; dilution 1:1000) was used as controls.

Changes in endothelial cell phenotype were examined by immuno- and affinity-fluorescence methods in HPAECs grown on Thermanox coverslips. Following fixation with 4% formaldehyde in PBS and permeabilization with 0.1% Triton X-100 in PBS, the cells were incubated with mouse monoclonal anti-CLIC4 (sc-135739, Santa Cruz Biotechnology; dilution 1:100), HA antibody (sc-805, Santa Cruz Biotechnology; dilution 1:100) or mouse monoclonal anti-VE cadherin antibodies (sc-9989, Santa Cruz Biotechnology; dilution 1:100). Specifically bound primary antibodies were localized using appropriate fluorescent-labelled secondary antibodies and F-actin was visualized with 1µg/ml TRITC-phalloidin (P1951, Sigma). Following immunostaining, cells were mounted in Vectashield and examined by fluorescent confocal microscopy (Leica, TCS SP5, Leica Biosystems, Bretton, Peterborough).
**Cell survival studies:** 60% confluent HPAECs grown in Corning® 96 well special optics plates were left untreated or were infected with adenoviruses, Ad Tet-off (AdControl), AdCLIC4 or Ad CLIC4 shRNA. The cells were cultured in full medium (non-starved controls) or in starvation medium deprived of serum and growth factors for 48 h r. Menadione (50µM, Sigma) was added to the cells grown in full medium for 6 hr to induce apoptosis (negative control). The cells were then incubated with MitoProbe™ DiOC2(3) (Life Technologies, Invitrogen,USA) for 45 minutes. DiOC2(3) penetrates the cytosol of eukaryotic cells and accumulates primarily in mitochondria with active membrane potentials, producing bright, red fluorescence. Images were taken under the under the Olympus IX70 inverted fluorescent microscope and F-view Soft Imaging System camera. The intensity of fluorescence corresponding to the number of live cells was measured with Image J.

**Cell metabolic activity:** A colorimetric assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Southampton, UK) was used to assess metabolic activity, the amount of absorbance at 490 nm being proportional to the number of living cells in culture.

**In vitro angiogenesis assays**

The angiogenic capacity of cultured endothelial cells was studied using a Matrigel tube formation assay (354230 [reduced serum] for HPAECs and 354234 on ECFCs; BD Biosciences). For the Matrigel assay, cells were trypsinized, re-suspended in growth factor-deprived media containing 1% FBS and seeded at a density of 7000 cells/well in triplicate in 96 well plates coated with 50 µl Matrigel. Plates were then incubated for 24 h, under normoxic or hypoxic conditions, prior to fixation in 4% formaldehyde in PBS and microscopic examination. Two phase-contrast images were captured from each well and total tube length determined using Image J software.
HIF expression, knockdown and stabilization

Expression of HIF-1α and HIF-2α was determined by western blotting. Proteins were separated by 4-12% NuPage® Bis-Tris gels (Invitrogen) using MOPS buffer at 200V constant for an hour (50 µg equivalent of protein per lane). Membranes were probed with a mouse monoclonal antibody (1:1000 dilution) against HIF-1α (OSA-602; Enzo Life sciences, Exeter, UK) or a mouse monoclonal antibody against HIF-2α (NB100-122, Novus Biologicals, Cambridge, UK). Controls included the use of mouse monoclonal antibodies against β-Actin (A2228, Sigma: 1:10,000 dilution). The relative intensity of the immunoreactive bands was determined by densitometry using ImageJ software.

Suppression of HIF-1α expression was achieved by transfecting HPAECs with siRNA for HIF-1α (siMax siRNA for HIF-1α, 5’-AGCAGUAGGAAUUGGAACAUU(dTdT); Eurofins MWG Operon, Ebersberg, Germany), or a scrambled sequence of similar GC content (Invitrogen) in OPTI-MEM medium (Invitrogen) using Oligofectamine (Invitrogen), according to the manufacturer's guidelines for transfecting HUVECs. The protein expression levels were measured 48 hours post-transfection. For experiments requiring adenoviral manipulation concurrent with siRNA knockdown, adenoviral transfections were performed 24 hours post-siRNA transfection and the cells were incubated for further 24 hours.

The stabilisation of HIF was studied using U2OS human osteosarcoma cells stably expressing a luciferase reporter construct under the control of a hypoxia response element (U2OS-HRE-luc), this being a kind gift from Dr M. Ashcroft, University College London. U2OS cells were left either untreated (control), infected with the CLIC4 adenovirus without the tet-off adenovirus (AdControl) or infected with CLIC4 and tet-off adenoviruses to allow expression
of CLIC4. The cells were then incubated under normoxic or hypoxic conditions for 24 hr and a luciferase assay (Promega) used to measure HIF activation.

**P65 expression, phosphorylation and NFκB activity measurement:** p65 expression and phosphorylation were analysed by Western blotting. The antibodies were from Cell Signaling Technology, Denver, USA (1:1000 dilution). AdNFκB-Luc, which contains the Luc gene driven by four tandem copies of the NFκB consensus sequence, was fused to a TATA-like promoter from the Herpes simplex virus thymidine kinase gene. The cells were infected with AdNFκB-Luc at the MOI 1:500 and luciferase expression was measured 24 hr post-transfection using a Luciferase Assay System (Promega)\textsuperscript{56}.

In order to verify the role of NFκB in the regulation of HIF activity, the NFκB inhibitor, BAY 117085 (10μM/L; Sigma) was added to the U2OS-HRE-luc cells 24 hr before luciferase assay.
**SUPPLEMENTAL TABLE**

Table S1. Patient and healthy volunteer data for the plasma CLIC4 study

<table>
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<td>PVR, dyn·s·cm⁻⁵ (Mean ± SD)</td>
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Table S2. Patient and healthy volunteer data for the endothelial progenitor study

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Data presented as median (range)
Figure S1. Immunostaining of endothelial markers in blood-derived endothelial cells from IPAH patients. Endothelial cells, derived from the peripheral blood of IPAH patients, display immunofluorescence staining for the endothelial markers A) CD31, B) VE-Cadherin, C) von Willebrand factor and D) UEA-1 lectin binding. Bar=20 µm.
Figure S2. CLIC4 protein expression in (A) kidneys and (B) right ventricles of pulmonary hypertensive rats. Representative Western blots are shown underneath the graphs. Data are presented as mean±SEM (n=3-6). *P<0.05; **P<0.01, comparison with untreated controls (Student’s t-test).
Figure S3. CLIC4 protein expression small intrapulmonary arteries of PH animals.

Changes in CLIC4 protein levels in intimal and medial layers of equally sized intrapulmonary arteries from the lung sections of control animals (C), chronic hypoxia PH animals (H), monocrotaline PH animals (M) and Sugen/hypoxia PH animals (S) were measured using Image J. Data are presented as mean±SEM (n=5). *P<0.05; ***P<0.001, comparison with intimal control; #P<0.05, comparison with medial control.
Figure S4. Expression of CLIC proteins in the wildtype (WT) and CLIC4 knockout (CLIC4 KO) mice. (A) Representative western blots showing differential expression of CLIC4 and the unchanged levels of its closely related proteins (CLIC1 and CLIC5) in the lungs of the WT and CLIC4 KO mice in normoxic and hypoxic conditions. β-actin staining served as a loading control. (B) Representative sections showing CLIC4 and von Willebrand Factor (vWF) staining in a pulmonary vessel of WT and CLIC4 KO mice.
Figure S5. The effects of hypoxia on CLIC4 localization and protein expression in HPAECs. (A) HPAECs were cultured in normoxia or were exposed to hypoxia (1 hr, 2% O₂). Arrow head indicates peripheral localization of CLIC4. Bar=10 µm. (B) Western blot images and densitometry results showing an increase in CLIC4 expression in HPAECs following 4-24 hr exposure to hypoxia that are prevented by ROS inhibitors, DPI (5 µmol/L) and rotenone (5 µmol/L). Data are presented as mean±SEM (n=3). ***P<0.001, compared with untreated controls.
Figure S6. CLIC4 expression in human pulmonary artery smooth muscle cells (HPASMCs) and endothelial cells (HPAECs) cultured in normoxic and in hypoxic conditions. Data are presented as mean±SEM (n=3). ***P<0.001, compared to normoxic HPASMC controls.
Figure S7. Morphological changes associated with CLIC4 expression in HPAECs.

Images show F-actin localization (left panel) and F-actin merged (red, right panel) with either GFP (B, F) or CLIC4 (D) (green). Adenoviral gene transfer was used to overexpress GFP (AdControl, A-B) or CLIC4 (C-D). Overexpression of CLIC4 (24 hr post-infection) was accompanied by membrane ruffling (arrow) whereas CLIC4 shRNA prevented the morphological changes associated with CLIC4 overexpression (E-F). Bar=10µm.
Figure S8. BMPR2 knockdown and CLIC4 expression in blood-derived endothelial cells and HPAECs. (A) Representative western blot showing BMPR2 and CLIC4 protein expression in cells derived from a healthy volunteer 2 days after transfection with scrambled siRNA control or BMPR2 siRNA. (B) Representative semi-quantitative RT-PCR image showing BMPR2 knockdown at the mRNA level. Transfection of ALK5 siRNA served as an additional negative control, not targeting BMPR2. β2-microglobulin was used as a reference gene. Densitometry of (C) BMPR2 and (D) CLIC4 protein expression, normalized to β-actin and relative to scrambled siRNA control. Quantitative RT-PCR assessment of (E) BMPR2 and (F) CLIC4 mRNA expression, normalized to PPIA and α-tubulin expression and relative to scrambled siRNA control. Densitometry of (G) BMPR2 and (H) CLIC4 protein expression, normalized to β-actin and relative to scrambled siRNA control. (I) Representative western blot showing BMPR2 and CLIC4 protein expression in cells derived from HPAECs 2 days after being transfected with scrambled siRNA control or BMPR2 siRNA. Data expressed as mean±SEM of cells derived from healthy volunteers (n=3 to 4). **P<0.01; ***P<0.001 (Student’s t-test).
Figure S9. VEGF and VEGFR-2 expression and VEGF-mediated CLIC4 effects on endothelial permeability in HPAECs. (A) Dot blot analyses showing increased expression of VEGF in HPAECs overexpressing CLIC4 both in normoxic and hypoxic conditions.
compared to control cells. (B) VEGFR-2 receptor expression was unchanged in HPAECs overexpressing CLIC4 (AdCLIC4). (C) CLIC4-induced endothelial permeability is prevented by function-blocking anti-VEGF antibodies. The cells were infected with adenoviruses (AdControl and AdCLIC4) 24 hr before permeability measurement. Control antibodies (dark grey bars) and VEGF-targeting (black bars) antibodies were added 2hr post-infection. Each bar represents mean±SEM, (n=4).
Figure S10. HIF-1α expression in HPAECs and mouse lungs. (A) Representative western blot showing reduced HIF-1α expression 2 days following transfection with HIF-1α siRNA. (B) Representative western blots showing differential expression of HIF-1α and HIF-2α in the lungs of the wildtype (WT) and CLIC4 KO mice in normoxic and hypoxic conditions. β-actin staining served as a loading control.
SUPPLEMENTARY REFERENCES


