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Endothelial ET_B Limits Vascular Remodelling and Development of Pulmonary Hypertension during Hypoxia

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Key Words
ET_B receptor · Knockout · Endothelin · Pulmonary hypertension

Abstract
Background: We hypothesised that the potential protective effects of endothelial ET_B are important in limiting pulmonary vascular muscularisation, vasoconstriction and the development of pulmonary arterial hypertension in response to hypoxia. ***Methods:*** EC-specific ET_B knockout mice (EC $ET_B^{-/-}$) and control mice ($ET_B^{f/f}$) were subjected to hypobaric hypoxic (10% FiO_2) or normoxic conditions for 14 days before assessment of right ventricular pressure and pulmonary vascular morphology and function. ***Results:*** During normoxia, no difference in right ventricular pressure was detected between EC $ET_B^{-/-}$ (23.7 ± 1.7 mm Hg) and $ET_B^{f/f}$ mice (20.2 ± 1.5 mm Hg). Hypoxia induced an exaggerated increase in right ventricular pressure in EC $ET_B^{-/-}$ mice (34.4 ± 1.2 mm Hg vs. 24.6 ± 1.4 mm Hg), accompanied by an increase in right ventricular mass. No effect was observed in $ET_B^{f/f}$ mice. Endothelin-1 constricted pulmonary arteries from both groups, although maximum response was similar irrespective of inspired oxygen or genotype. Hypoxia increased the percentage of muscularised vessels in both groups of mice, but the percentage increase was significantly greater in EC $ET_B^{-/-}$ mice. ***Conclusions:*** The potential protective effects of endothelial ET_B are important in limiting pulmonary vascular muscularisation and the development of pulmonary arterial hypertension in response to hypoxia.

Introduction

Pulmonary arterial hypertension (PAH) is a progressive condition involving small pulmonary arteries (PAs) characterised by a sustained increase in pulmonary vascular resistance and vascular remodelling leading ultimately to right ventricular failure and premature death [1]. Several lines of evidence implicate endothelin-1 (ET-1) in the aetiology and progression of PAH. First, ET-1 is a potent vasoconstrictor and mitogen [2]. Second, plasma concentration of ET-1 correlates with severity of PAH in animal models [3–5] and patients [6], and third, non-selective and selective endothelin A receptor antagonists improve symptoms and slow the progression of PAH [7, 8]. The lung is an important site of ET-1 production with a concentration of ET-1 five times greater than that seen in other organs [9]. Endothelin B receptor (ET_B) mRNA is expressed abundantly in the lung [10], particularly in distal segments of the pulmonary vascular tree [11], where

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it is found in the endothelium and media of pulmonary blood vessels as well as in bronchioles and alveoli.

In contrast, endothelin A receptors (ET_A) are localised to the media of large proximal PAs and veins with relatively little expression in distal arterioles [11–13]. Hypoxia increases the expression of ET-1, ET_A and ET_B throughout the rat lung [10, 12] with histological evidence to suggest a preferential increase in endothelial cell (EC) ET_B expression in distal segments [13]. EC ET_B elicit vasodilatation and anti-mitogenic effects through the release of nitric oxide (NO) and/or prostaglandin I_2 (PGI_2) [14, 15]. Increased ET-1/EC ET_B-mediated vasodilatation has thus been hypothesised to protect against hypoxia-induced vasoconstriction [13], and studies in rats suggest that ET_B deficiency exacerbates monocrotaline- [16] and hypoxia-induced PAH [17]. Pulmonary ET_B also clear ET-1 from the plasma [18], further limiting ET_A-mediated vasoconstrictor and mitogenic effects.

Vascular smooth muscle cell (VSMC) ET_B mediate vasoconstriction in humans [19]. In hypoxic rat models [20] and sheep models of embolism-induced PAH and PAH secondary to aortopulmonary shunting, there is evidence of increased VSMC ET_B-mediated vasoconstriction [21, 22]. Activation of ET_A and ET_B on VSMC by ET-1 also promotes cellular hypertrophy [11] and hence may promote the progression of PAH through muscularisation of small pulmonary arterioles. Thus, with respect to the development of PAH, pulmonary ET_B have the potential to elicit both protective and detrimental effects. Study of the interplay between EC and VSMC ET_B is likely to increase our mechanistic understanding of the role of the endothelin system in the pathogenesis of PAH. We have previously generated EC-specific ET_B knockout (KO) mice that exhibit endothelial dysfunction in the absence of systemic hypertension, with evidence of impaired endogenous NO release and increased plasma ET-1 [23]. Otherwise, they have normal feeding and growth rates, exhibit normal behaviour and are healthy. Here we have used this model to determine whether the potential protective effects of EC ET_B are important in limiting pulmonary vascular muscularisation and the development of PAH during hypoxia.

Methods

Experimental Animals

Mice featuring selective KO of EC ET_B were generated using a Cre-*LoxP* approach as previously described [23]. Briefly, mice with *loxP* sites flanking exons 3 and 4 of the ET_B gene ('floxed' mice, $ET_B^{f/f}$) were crossed with mice expressing a Cre recombinase transgene in an EC-restricted pattern (WW/Tie2-Cre) [24] to produce EC-specific ET_B KO mice (EC $ET_B^{-/-}$). Genotyping to identify the flox and recombined alleles was performed by Southern blot and by PCR using primers amplifying a sequence spanning the 3′ and 5′ *loxP* sites (forward primer: TCA GTT GTA ATG AGA CAC AGA C; reverse primer: AGC CAT AAA GTC ACA GCC ATT C). The Tie2-Cre transgene was detected by PCR as described [24]. Male mice aged 8–12 weeks (weight 25–35 g) were studied and EC $ET_B^{-/-}$ mice compared with $ET_B^{f/f}$ control mice in all experiments. The genetic background of each group was 129/Ola; BKW; C57Bl/6; $SJLF_1$. All procedures were carried out with the approval of the University of Glasgow and University of Edinburgh Local Ethical Review Committees, under Home Office Project and Personal Licence authority.

Hypobaric Chambers

EC $ET_B^{-/-}$ mice and control mice (10 animals/group) were exposed to hypobaric hypoxic conditions for 14 days by housing them in a specially designed hypobaric chamber, as previously described [20]. The chamber was depressurised over the course of 2 days to 550 mbar [55 kPa or 413 mm Hg, equivalent to FiO_2 (percentage oxygen in inspired air) = 10%]. A further 10 age-matched mice of each genotype were maintained in the same room breathing air at atmospheric pressure (FiO_2 = 21%). All mice were allowed free access to standard rodent chow and water throughout the study and kept under 12-hour light/dark cycles.

Haemodynamic Studies

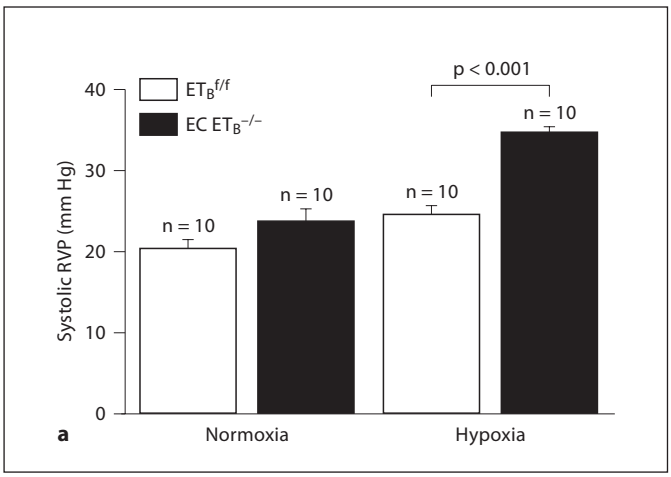
Anaesthesia was induced with 2–4% halothane and maintained with 1.5% halothane using a mix (1 part:3 parts) of NO_2 and high-flow O_2. Pressure and heart rate measurements were performed and analysed as previously described [25]. Systemic arterial pressure was measured via a cannula (Portex, 0.75 mm OD) inserted into the right carotid artery. A 25-gauge needle was advanced into the right ventricle (RV) via a transdiaphragmatic approach for measurement of right ventricular pressure (RVP). At the end of the experiment, mice were killed by lethal overdose of halothane. The heart was removed, blotted dry of blood and weighed. The right lung was placed in formal saline (1 part 37% formaline:9 parts 0.9% saline) for histology and third-order PAs dissected from the left lung for wire myography experiments.

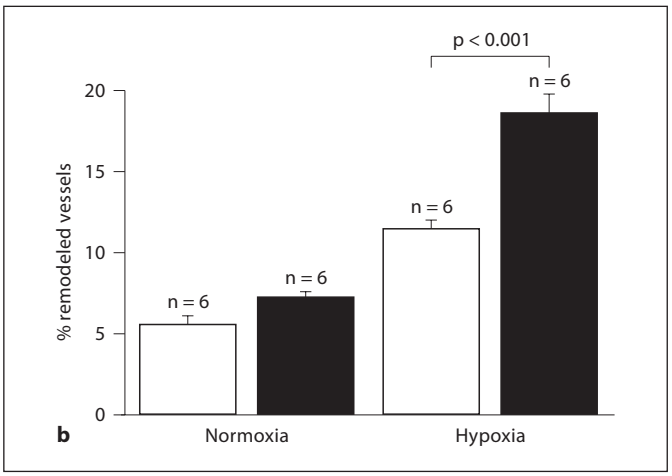
Assessment of RV Hypertrophy

Hearts were dissected clean of pericardial tissue, blotted dry and the atria and great vessels removed to the plane of the atrioventricular valves. The RV free wall was dissected free from the left ventricle and septum (LV+S) and weighed. The ratios of RV/body weight (BW), RV/(LV+S) and RV/total ventricles (TV) were calculated [25].

Lung Histology

The right lung was embedded in paraffin and 10-μm sections stained with Miller's elastin stain [26] and with picrosirius red for collagen [27]. Sections were microscopically assessed for muscularisation of small PAs (25–100 μm external diameter) associated with an airway distal to the respiratory bronchiole. Arteries were considered muscularised if they possessed a distinct double-elastic lamina visible for at least half the diameter of the vessel in cross-section (fig. 2). The percentage of vessels containing double-elastic lamina was calculated as the number of muscularised





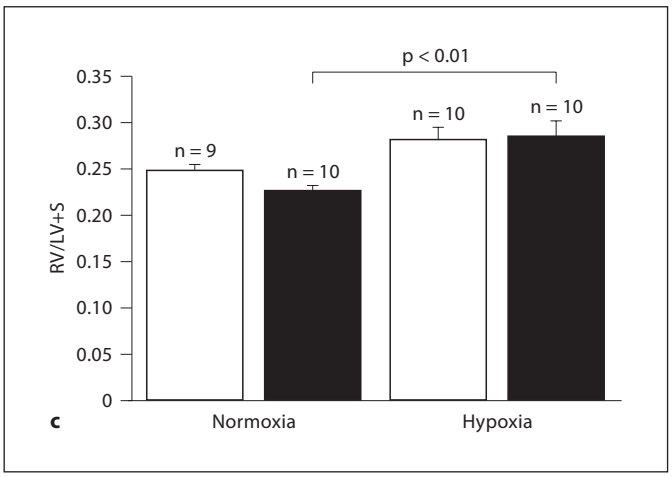


Fig. 1. a Systolic right ventricular pressure of anaesthetised $ET_B^{f/f}$ and EC $ET_B^{-/-}$ mice (n = 10). **b** Percentage of muscularised vessels in lungs from EC $ET_B^{-/-}$ mice and $ET_B^{f/f}$ controls (n = 6) following 14 days of normoxia or hypobaric hypoxia. **c** Right ventricular hypertrophy, as determined by the ratio RV/(LV+S), of $ET_B^{f/f}$ and EC $ET_B^{-/-}$ mice (n = 10).

vessels/total number of vessels counted per section × 100. Three sections from each right lung were assessed for every mouse. A total of 6 mice per group were analysed.

In vitro Wire Myography

Third-order PAs (first interlobar; approx. 300 μm internal diameter) from the left lung were cut to yield two 2 mm-long segments, which were then mounted onto a wire myograph. Vessels were bathed in Krebs buffer solution (118.4 mM NaCl; 25 mM $NaHCO_3$; 4.7 mM KCl; 1.2 mM KH_2PO_4; 0.6 mM $MgSO_4$; 2.5 mM $CaCl_2$; 11 mM glucose; pH 7.4) at 37°C and constantly bubbled with 16% O_2/5% CO_2. Tension was applied to give transmural pressures equivalent to 12–14 mm Hg for controls and 30–33 mm Hg for hypoxic animals. These pressures are similar to those experienced by pulmonary vessels in vivo in rodents under similar hypobaric conditions [28, 29]. Following equilibration, PA rings were constricted twice with 50 mM KCl solution. Cumulative concentration-response curves were constructed for ET-1 (10^{-15} to 10^{-7} M) following a 30-min incubation with 100 μM N-nitro-L-arginine methylester (L-NAME) or vehicle. All responses were expressed as a percentage of the maximal KCl-induced constriction.

Drugs

Halothane, formalin and L-NAME were all purchased from Sigma-Aldrich (Gillingham, UK). ET-1 was purchased from Merck Chemicals Limited (Nottingham, UK).

Data Analysis and Statistical Procedures

Data are expressed as means ± SEM. Statistical comparisons were made by two-way ANOVA. When significance was attained ($p < 0.05$), differences were established using the Bonferroni multiple comparison test. In the myography studies, pEC_{50} values were calculated from concentration-response curves by graphical interpolation (GraphPad Prism 4.0).

Results

Haemodynamic Studies

Systemic mean arterial blood pressure was not significantly different between EC $ET_B^{-/-}$ mice and controls under either normoxic ($ET_B^{f/f}$ 99 ± 4 mm Hg; EC $ET_B^{-/-}$

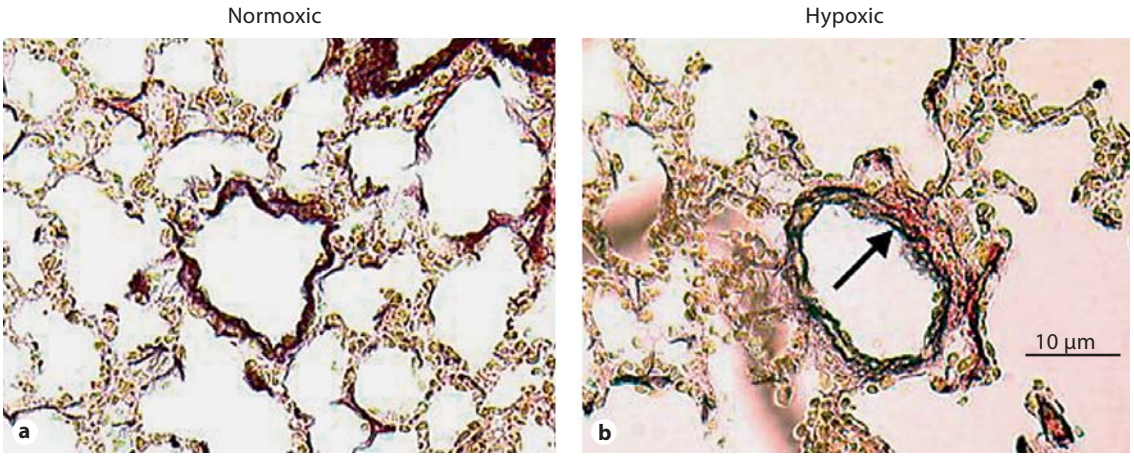


Fig. 2. Representative sections illustrating normal murine small PAs under normoxic conditions (**a**) and muscularised arteries with a double elastic lamina (**b**, arrow) that develop following 14 days of hypobaric hypoxia. The proportion of muscularised to normal vessels in response to hypoxia was greater in EC $ET_B^{-/-}$ mice than in $ET_B^{f/f}$ mice. Sections are stained with Miller's elastin stain and picrosirius red collagen stain.

Table 1. Indices of right ventricular hypertrophy in $ET_B^{f/f}$ and EC $ET_B^{-/-}$ mice under normoxic conditions or following 14 days of hypobaric hypoxia

	$ET_B^{f/f}$			EC $ET_B^{-/-}$		
	RV/(LV+S)	RV/TV	RV/BW	RV/(LV+S)	RV/TV	RV/BW
Normoxic	0.25 ± 0.009	0.20 ± 0.006	0.80 ± 0.065	0.22 ± 0.009	0.18 ± 0.006	0.75 ± 0.047
Hypoxic	0.28 ± 0.012	0.22 ± 0.007	1.03 ± 0.083*	0.29 ± 0.017*	0.22 ± 0.010*	0.98 ± 0.045*

Values are means ± SEM (10 animals/group). * $p < 0.05$ for comparison between normoxic and hypoxic animals within each genotype.

101 ± 4 mm Hg) or hypoxic conditions ($ET_B^{f/f}$ 93 ± 4 mm Hg; EC $ET_B^{-/-}$ 103 ± 4 mm Hg), as previously reported [23]. No difference in heart rate was observed. Although systolic RVP was similar between genotypes under normoxic conditions, following 2 weeks of hypoxia, systolic RVP in EC $ET_B^{-/-}$ mice was significantly elevated compared to hypoxic $ET_B^{f/f}$ controls (fig. 1a).

Right Ventricular Hypertrophy

Relative right ventricular mass, as measured by RV/(LV+S) and RV/TV ratios, tended to be lower in normoxic EC $ET_B^{-/-}$ mice compared to normoxic $ET_B^{f/f}$ controls. Body weight fell by approximately 3.4 g in both genotypes following 2 weeks of hypoxia. Both genotypes demonstrated a significant increase in RV/BW ratio when exposed to hypoxia. However, only hypoxic EC $ET_B^{-/-}$ mice demonstrated a significant increase in RV/(LV+S) ratio and RV/TV, suggesting a preferential increase in RV mass (fig. 1c; table 1).

Vascular Morphology

Under normoxic conditions, the percentage of muscularised vessels was similar in EC $ET_B^{-/-}$ and $ET_B^{f/f}$ mice. Following hypoxia, muscularisation was observed in a significantly greater proportion of vessels from EC $ET_B^{-/-}$ mice compared to controls (fig. 1b, 2).

Myography

Neither genotype nor FiO_2 influenced the E_{max} or pEC_{50} of PAs to ET-1 (table 1). Treatment with L-NAME did not significantly alter the maximum constriction or tissue sensitivity to ET-1 in either genotype (table 2).

Table 2. Potency and maximum effect of ET-1 in PA rings from EC $ET_B^{-/-}$ and $ET_B^{f/f}$ mice

	$ET_B^{f/f}$			EC $ET_B^{-/-}$		
	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n
Normoxic	9.2 ± 0.1	121.9 ± 4.8	7	8.8 ± 0.1	109.7 ± 7.2	7
Hypoxic	10.1 ± 0.2	116.8 ± 9.0	5	9.6 ± 0.1	125.4 ± 5.5	5
Normoxic + L-NAME	9.7 ± 0.2	126.3 ± 3.4	7	9.1 ± 0.9	120.2 ± 5.0	6
Hypoxic + L-NAME	10.1 ± 0.2	124.3 ± 7.1	6	10.5 ± 0.1	143.0 ± 6.0	9

E_{max} is expressed as a percentage of the maximal contractile response to 50 mM KCl solution.

Discussion

This study demonstrates that in response to 14 days of hypobaric hypoxia, selective loss of EC ET_B results in an exaggerated increase in systolic RVP, an increase in RV mass and an increase in the proportion of muscularised small PAs. However, we did not observe systemic hypertension, implying a selective effect of loss of EC ET_B in the pulmonary vasculature.

After 14 days of hypoxia, we observed no increase in systolic RVP in control $ET_B^{f/f}$ mice that were of the same genetic background as EC $ET_B^{-/-}$ mice. The development of hypoxia-induced PAH differs between mice of different strains, although even relatively resistant mice have been shown to develop raised systolic RVP after 4 weeks of hypoxia [30]. It is likely that our control $ET_B^{f/f}$ mice would have developed elevated systolic RVP after such a prolonged period of hypoxic exposure. However, we can conclude that loss of EC ET_B either accelerated the increase in systolic RVP in this strain or enabled PAH to develop in a strain resistant to the effects of hypoxia.

There are several possible mechanisms that may underlie the exaggerated increase in RVP in EC $ET_B^{-/-}$ mice. First, EC ET_B vasodilator pathways are likely to be an important protective mechanism that limits the development of PAH during chronic hypoxia. In vitro studies of rat pulmonary microvascular ECs demonstrate that an increase in shear stress increases ET_B expression and enhances ET-1-mediated ET_B-dependent eNOS activation [31]. Lungs from rescued ET_B-deficient rats also demonstrate an exaggerated pressor response to ET-1 [32], due in part to reduced NO and PGI_2 production [17]. Studies of eNOS over-expressing [33] and eNOS KO mice [34, 35] have revealed that a reduction in EC-derived NO increases vascular tone and muscularisation of PAs. We have previously reported endothelial dysfunction with decreased NO bioavailability in the absence of systemic hypertension in the aortae of EC $ET_B^{-/-}$ mice [23]. Thus, impaired EC ET_B-mediated NO/PGI_2 release from pulmonary resistance vessels during hypoxia may have contributed to the development of PAH. We also observed an exaggerated increase in the number of muscularised small pulmonary vessels in EC $ET_B^{-/-}$ mice following hypoxia. Thus, loss of ET_B-mediated NO release may also have had a permissive effect on hypoxia-induced vessel muscularisation.

Second, EC $ET_B^{-/-}$ mice have elevated plasma ET-1 concentration [23] that may directly exert pressor and mitogenic actions to further promote the development of PAH. Studies in our laboratory demonstrate that clearance of ET-1 is impaired and plasma ET-1 increased approximately 4-fold in these mice [23]. Increased plasma ET-1 has also been reported in patients with PAH [6, 36, 37], though this may reflect increased production rather than impaired pulmonary clearance [38]. Loss of ET_B signalling has also been reported to increase ECE-1 mRNA expression [39], which may further contribute to an increase in ET-1. However, the limited experimental evidence available suggests that an isolated increase in ET-1 is insufficient to cause PAH. Under normoxic conditions, rats chronically infused with ET-1 by subcutaneous pump do not develop raised systolic RVP [40]. Similarly, transgenic mice that over-express preproET-1 have elevated plasma ET-1, exhibit pulmonary inflammation and fibrosis, but do not develop PAH, even when exposed to mild hypoxia (FiO_2 16%) [41]. Thus, although an increase in ET-1 may contribute to many of the pathological processes associated with PAH, concomitant loss of NO/PGI_2-mediated vasodilator pathways is likely to be necessary for PAH to develop.

Although expression of Tie2 was thought to be exclusively restricted to ECs, Tie2-positive monocytes have now been identified [42, 43]. Thus, Cre-Lox-mediated ablation of the ET_B gene may have occurred in such inflammatory cells, as well as in ECs, potentially complicating the interpretation of the phenotype of the EC $ET_B^{-/-}$ mice. Although no inflammatory infiltrate was seen in the lung sections from the hypoxic EC $ET_B^{-/-}$ mice, further studies to clarify the role of such macrophages in the development of PAH are required.

We found no difference in the maximal response or sensitivity of 3rd order PAs to ET-1 in EC $ET_B^{-/-}$ mice. The absence of any change in ET-1-mediated constriction following L-NAME suggests that NO does not contribute significantly to vascular tone in vessels of this size. This finding does not necessarily contradict our hypothesis that loss of ET_B-mediated NO release contributed to hypoxia-induced PAH in EC $ET_B^{-/-}$ mice. Indeed, studies of rat intrapulmonary arteries following 4 h of either normoxia or hypoxia found similar results, demonstrating no effect of nitro-L-arginine on ET_B-mediated contraction [44]. In contrast, chronic in vivo telemetric measurements of PAP demonstrate that L-NAME acutely increases PAP in the mouse [45], suggesting that NO is an important determinant of PAP in this species. Thus, hypoxia-induced PAH in EC $ET_B^{-/-}$ mice may be due to an increase in the tone of resistance arterioles smaller than those possible to study in myography experiments.

Several studies in mice report weight loss following exposure to hypoxia, an effect that is incompletely understood but may involve altered expression of genes regulated by hypoxia-inducible factor α [46]. However, we saw no difference in weight loss (approx. 3.4 g) between controls and EC $ET_B^{-/-}$ mice. Although weight loss contributed to the increase in RV/BW ratio in both groups, we only observed an absolute increase in RV mass [as determined by RV/(LV+S) ratio and RV/TV ratio] in EC $ET_B^{-/-}$ mice. This increase is, therefore, likely to reflect a true RV hypertrophic response to the increased PAP in EC $ET_B^{-/-}$ mice, rather than any disproportionate weight loss in KOs.

In conclusion, this study indicates that EC ET_B play an important protective role during prolonged hypoxia that limits vascular remodelling and the development of pulmonary hypertension.

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