Early View

Original article

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Summary: Activation of HIF2 by hypoxia initiates vascular cell proliferation and recruitment of inflammatory cells at early stages of PH development through HIF2-dependent transcription of genes involved in these pathways in pulmonary vascular cells.

ABSTRACT

Most published studies addressing the role of HIFs in hypoxia-induced PH development employ models that may not recapitulate the clinical setting, including the use of animals having pre-existing lung/vascular defects secondary to embryonic HIF ablation or activation. Further, critical questions including how and when HIF-signaling contributes to hypoxia-induced PH remains unanswered.

Normal adult rodents in which global HIF1 or 2 was inhibited by inducible gene deletion or pharmacological inhibition (antisense oligonucleotides-ASO and small molecule inhibitors) were exposed to short-term (4 days) or chronic (4-5 weeks) hypoxia. Hemodynamic studies were performed, the animals euthanized and lungs and heart obtained for pathologic and transcriptomic analysis. Cell-type specific HIF signals for PH initiation were determined in normal pulmonary vascular cells in vitro and in mice (using cell-type specific HIF deletion).

Global HIF1α deletion in mice did not prevent hypoxia-induced PH at 5 weeks. Mice with global HIF2α deletion did not survive long-term hypoxia. Partial HIF2α gene deletion, or HIF2-ASO (but not HIF1-ASO) reduced vessel muscularization, rises in pulmonary artery pressures and right ventricular hypertrophy in mice exposed to 4-5 week hypoxia. A small molecule HIF2 inhibitor (PT2567) significantly attenuated early events (monocyte recruitment and vascular cell proliferation) in rats exposed to 4-day hypoxia as well as vessel musculization, tenascin C accumulation and PH development in rats exposed to 5 week hypoxia. In vitro, HIF2 induced a distinct set of genes in normal pulmonary vascular EC, mediating inflammation and proliferation of EC and SMC. EC HIF2α knockout prevented hypoxia-induced PH in mice.

Inhibition of HIF2, not HIF1 can provide a therapeutic approach to prevent the development of hypoxia-induced PH. Future studies are needed to investigate the role of HIFs in PH progression and reversal.

INTRODUCTION

Pulmonary hypertension due to lung disease and/or hypoxia (WHO group 3 PH) comprises nearly one million people worldwide making it the second largest group of patients suffering from this disease. Unfortunately, none of the currently approved drugs for group 1 PAH have been shown in randomized controlled trials to benefit patients with group 3 PH, emphasizing the need for a better understanding of disease mechanisms as they might aid in the discovery of new therapies.

There is abundant evidence supporting the central involvement of the hypoxia inducible factors (HIFs) in chronic hypoxia-induced PH (1-11). However, we believe the experimental approaches used in most previously published studies may not recapitulate the clinical settings. For example, some group 3 PH development is due to hypoxia exposure in post-natal humans and animals with normal pulmonary circulations. It is important to note that except for two studies in which Hif1 deletion in SMC or EC was initiated in adult mice (10, 11), in all other studies Hif1α or Hif2α deletion was initiated in developing embryos (1-9). It has been well-established that all these HIF knockout or activation models (PHD2 KO) exhibit vascular defects in developing embryos and likely in adult mice derived from these embryos (12-17). Also, due to the essential role of HIF in development, most published studies use cell-type specific HIF knockout models (2-7, 10, 11). We believe such cell-type specific HIF knockout approaches may have shortcomings for the purpose of determining the general function of a gene like HIF in a disease such as PH where interactions between multiple cell types (EC, SMC, and Fibs) are clearly necessary for disease progression and a specific gene may have different or opposite functions in different disease-involved cell types.

Beside inducible deletion, pharmacologic inhibition could be an important tool to study the general role of HIF in PH development in normal adult animals. Given the potential important role of HIF proteins and particularly HIF2 in PH, successful pharmacologic inhibition could also lay an important foundation for potential PH treatment. Recent studies have led to small molecules that specifically block HIF2, but not HIF1 activity (18, 19). These inhibitors have also been shown to exhibit strong antitumor activity in vivo and in vitro (18, 20). Additionally, antisense oligonucleotides (ASOs) to Hif1 and 2 have also been developed and shown to attenuate HIF expression in vivo and to abrogate HIF mediated disease pathology (21).

In the study presented here, we sought to re-evaluate the role of HIFs in hypoxia-induced PH using approaches that may better recapitulate the clinical settings. Specifically, HIF activity is inhibited by inducible gene deletion and pharmacologic inhibitors in which global (not cell-type specific) HIF1 or HIF2 activity inhibition is initiated in normal adult animals without any predisposed diseases resulting from embryonic deletion of HIFs. In addition, we sought to better understand the underlying molecular mechanisms concerning how HIFs promote PH development, by studying the role of HIF in early as well as late stages of PH development.

RESULTS

Hif1α is dispensable in establishing hypoxia-induced PH at 5 weeks in adult mice

To delete the Hif1α gene in adult mice globally, Hif1α$^{fl/fl}$;UbcCreERT$^+$ and Hif1α$^{fl/fl}$;UbcCreERT$^-$ (control) mice were injected with tamoxifen for 5 days. Mice were then allowed to rest for one week, before exposure to either hypobaric hypoxia (P$_B$=370mmHg) or normobaric normoxia (P$_B$=740mmHg) for 5 weeks (Fig 1A). After five weeks hypoxia exposure, readout parameters were measured and animals were euthanized. The efficiency of Hif1a gene deletion in UbcCreERT$^+$ mice under hypoxic conditions is at 80% (Suppl. Table 1). Hypoxic exposure induced increases of hematocrit (Fig 1B), right ventricular systolic pressure, RVSP (Fig 1C), and right ventricular hypertrophy, as indicated by increases in Fulton index (Fig 1D) and in the ratio of the weight of the right ventricle (RV) versus body mass (weight$_{RV}$/weight$_{Body}$) (Fig 1E) in both Hif1α$^{fl/fl}$;UbcCreERT$^-$ and Hif1α$^{fl/fl}$;UbcCreERT$^+$ mice. Hypoxia also similarly reduced body weights and increased heart rate in both UbcCreERT$^+$ and UbcCreERT$^-$ animals, compared to normoxic animals (Suppl. Table 2). Thus, our data demonstrate that Hif1α expression is dispensable for development of PH and RV hypertrophy in normal adult mice after 5 weeks of hypoxia.

Mice with global genetic deletion of Hif2α do not survive long-term hypoxia

To investigate the role of *Hif2α* in hypoxia-induced PH development in normal adult mice, adult Hif2α$^{fl/fl}$;UbcCreERT$^+$ and Hif2α$^{fl/fl}$;UbcCreERT$^-$ mice were similarly treated with tamoxifen and exposed to normoxia or hypoxia, as we did for *Hif1α* mice. The efficiency of Hif2a gene deletion in hypoxic Hif2α$^{fl/fl}$;UbcCreERT$^+$mice, at the end of the experiment was at 71% (Suppl. Table 1). While there was no difference in survival and

PH development among Hif2α$^{fl/fl}$;UbcCreERT$^-$ mice, Hif1α$^{fl/fl}$;UbcCreERT$^+$ mice and Hif1α$^{fl/fl}$;UbcCreERT$^-$ animals (Fig 1 and Fig 2A), all Hif2α$^{fl/fl}$;UbcCreERT$^+$ mice died within 4 weeks of hypoxic exposure (Fig 2A). This data indicates that global Hif2α, but not Hif1α, deletion is not compatible with survival of mice exposed to chronic hypoxic conditions.

Global partial Hif2α deletion diminishes development of hypoxia-induced PH at 5 weeks in adult mice

To prevent lethality, we generated Hif2α$^{wt/fl}$;UbcCreERT$^+$ and Hif2α$^{wt/fl}$;UbcCreERT$^-$ mice, then treated them with tamoxifen and exposed them to hypoxia for 5 weeks. The efficiency of Hif2a gene deletion in hypoxic Hif2α$^{wt/fl}$;UbcCreERT$^+$mice at the end of the experiment was 36% (Suppl. Table 1). Cre-negative animals displayed increases in hematocrit (Fig 2B), RVSP (Fig 2C), Fulton index (Fig 2D), and weight$_{RV}$/weight$_{Body}$ ratio (Fig 2E) in response to hypoxia. While there were still significant increases in these parameters in hypoxia-exposed Hif2α$^{wt/fl}$;UbcCreERT$^+$ mice, the level of increase for some parameters was significantly reduced in comparison to cre-negative littermates (Fig 2D-E). Thus, our data, generated from normal adult mice, demonstrate that reduction of Hif2α attenuates development of PH induced by 5 weeks of hypoxia exposure.

Knockdown of Hif2α utilizing antisense-oligonucleotides significantly reduces development of hypoxia-induced PH at 5 weeks in adult mice

To further investigate the role of Hif2α in hypoxia-induced PH in normal adult mice and to examine whether targeting Hif2α therapeutically in adult mice under hypoxic conditions would be beneficial or lead to lethality, we used an antisense-oligonucleotide

(ASO) approach. The effectiveness and feasibility of the Hif2α ASO in reducing Hif2α expression in adult mice maintained under normoxia, was tested in a pilot experiment, which showed an up to 90% reduction of Hif2α mRNA levels in lungs, spleens, kidneys and livers in mice treated with Hif2α-ASO two times per week for two weeks (Fig 3A left), without significant changes of Hif1α mRNA in all organs examined (Fig 3A right). Further, Hif2α-ASO also reduced expression of the HIF2 target gene Epo1, but not the HIF1 target gene Pgk1 in kidneys (Fig 3B).

To determine the general role of HIF in hypoxia-induced PH development, mice were injected with Hif2α-ASO, or a Control-ASO (unspecific, no known target) for one week with two injections per week and then exposed to either normoxia or to hypoxia for five weeks during which two injections of ASO per week were maintained (Fig 3C). The efficiency of Hif2a gene deletion in hypoxic mice targeted with Hif2a ASO was 72% at the end of the experiment (Suppl. Table 1). We did not observe any lethality in Hif2α-ASO treated mice under hypoxia. However, hypoxic Hif2α-ASO treated mice exhibited a trend towards increased weight loss (Suppl. Table 4). Further studies also revealed that levels of circulating catecholamines (epinephrine and norepinephrine) were reduced by HIF2α ASO treatment as were heart rate, cardiac output and dP/dt $_{max}$ (Suppl. Fig. 2B-E). In addition, two animals died during readout procedures. All these suggested an increased fragility in these mice.

The hematocrit in Hif2α-ASO treated mice was lower, both, under normoxia and under hypoxia (Fig 3D). Under hypoxic conditions, Hif2α-ASO-treated mice also exhibited reduction in RVSPs (Fig 3E), Fulton index (Fig 3F), and weight$_{RV}$/weight$_{Body}$ ratio (Fig 3G), compared with hypoxic Ctrl-ASO mice. Consistent with hemodynamic

data, hypoxia exposed Hif2α-ASO-treated animals exhibited a marked reduction in fully muscularized vessels in the lungs, compared to hypoxia exposed Ctrl-ASO mice (Fig 3H-J).

On the other hand, the Hif1α-ASO reduced Hif1α mRNA by 60% in lung tissues (Suppl. Table 1), but it had no effect on Hct, RVSP, or the Fulton index in hypoxic mice (Fig 3K), similar to the lack of effect observed in inducible Hif1α KO mice (Fig 1).

A small molecule Hif2 inhibitor PT2567 significantly reduces development of hypoxia-induced PH at 4 weeks in adult rats.

To further address the safety of HIF2 inhibition and the role of HIF2 in initiation of hypoxia-induced PH, we treated rats (which develop more severe PH than mice) with a small molecule inhibitor (Peloton, PT2567) that specifically blocks HIF2 activity (18, 19). 4 week hypoxia exposure led to PH development in rats treated with the control reagent (0.5% methylcellulose and 0.5% Tween-80), as demonstrated by increased mean Pulmonary Arterial Pressure (mPAP) (Fig 4A) and increased Fulton Index (Fig 4B). The HIF2 inhibitor, PT2567, reduced the mPAP (Fig 4A) and attenuated RV remodeling (Fig 4B). To better understand the molecular mechanisms underlying HIF2's role in PH development in adult animals, we examined multiple pathways and genes that are important in PH development (3-7, 22-24). Indeed, increased inflammatory cell accumulation (monocytes) (Fig 4C, top panel), slight increased number of proliferating cells (Ki67) (Fig 4C, middle panel) and increased Tenascin C (TNC) expression (Fig 4C, lower panel) were observed in hypoxia-exposed rats, which were reduced by the HIF2 inhibitor (PT2567) in adventitial areas of pulmonary vessels from hypoxia exposed rats. The changes in proliferation and TNC expression in hypoxic rats and rats treated with

HIF2 inhibitor using immunostaining were confirmed by CCNA1 (a cyclin, marker for cell proliferation) and TNC mRNA levels in lung tissues of the corresponding rats (Fig. 4C middle and bottom right). However, the increased monocyte accumulation in hypoxic rats using immunostaining was not confirmed by their mRNA levels in lung tissues of the corresponding rats (Fig 4C, top right), likely due to the possibility that macrophages/monocytes are attracted to pulmonary vessels, but the total cell numbers are not significantly altered in the whole lungs of rats exposed to hypoxia for 4 weeks. In addition, more muscularized vessels were observed in hypoxia-exposed rats, which were reduced by the HIF2 inhibitor (PT2567) (Fig 4D and E), data consistent with our HIF2α ASO observations in mice. Further, consistent with previous reports (3-7, 23), a number of functionally important genes in PH such as Icam1, Sdf1, Arg1, Arg2, Ccnd1, Edn1, Pai1, Tgfa and Tsp1 exhibited increased levels in lungs of hypoxia exposed rats (Suppl. Fig 3). The increase in the above genes, but not genes such as Adm, Glut1, and Ndrg1 was significantly reduced in hypoxia exposed rats treated with the HIF2 inhibitor PT2567 (Suppl. Fig 3), suggested that the first group of genes likely represents target (direct or indirect) genes unique to HIF2, while the HIF2 inhibitor insensitive genes are likely to be regulated in a Hif2-independent manner at this time point. These data indicate that HIF2 inhibition significantly reduces development of hypoxia-induced PH, by preventing induction of genes involved inflammation (Suppl Fig 3B) and cell signaling (proliferation and fibrotic responses) (Suppl Fig 3C).

Hif2 activity is required for increased* accumulation of monocytes and increased cell proliferation *observed in hypoxia-exposed adult rats at 4 days.

Using both genetic and pharmacological approaches, our studies shown above demonstrated that inhibtion of HIF2 is able to attenuate the development of PH at 4-5 weeks of hypoxia using normal adult mice and rats as models. Studies below are intended to address multiple critical remaining questions concerning how HIF2 promotes PH initiation, but not to determine the general role of HIF2 in hypoxia-induced PH. The first question we wanted to answer is at which stage of PH development HIF2 signaling has an impact. To help address this question, we performed short-term hypoxia experiments. 4-day hypoxia exposure led to moderate increases of mPAP (Fig 5A) and Fulton index (Fig 5B) in rats treated with control reagent, in which only the Fulton index was reduced by the HIF2 inhibitor PT2567 (Fig 5B). Consistent with our previous findings (25, 26), in short-term hypoxia exposed rats, changes in TNC expression were minimal (Fig 5C), but there was a significant increase in accumulation of monocytes and cell proliferation (Fig 5C). The HIF2 inhibitor PT2567 completely abolished these changes (Fig 5C). The changes of Cd68, CCNA1 and TNC expression in hypoxic rats and rats treated with HIF2 inhibitor examined by immunostaining, were confirmed by their mRNA levels in lungs of the corresponding rats (Fig. 5C right side). Examination of gene expression in lungs demonstrated that most HIF2 regulated genes (Icam1, Sdf1, Arg1, Arg2, Ccnd1, Edn1, Pai1, but not Tgfa and Tsp1) that were observed in 4 week hypoxia rats (Suppl. Fig 3), also exhibited induction in 4 day hypoxia exposed rats (Suppl. Fig 4). Importantly, the Hif2 inhibitor PT2567 reduced expression of these genes (Suppl Fig 4). However, Pdgfb and CXCR4 were induced, and Id1 was reduced, only in rats exposed to short-term hypoxia (Suppl. Fig 4). The effectiveness of HIF2 inhibitor in preventing monocyte/macrophage accumulation and

vascular cell proliferation, and the fact that changes in gene expression were largely overlapping in rats exposed to short-term hypoxia and to long-term hypoxia, support the idea that HIF2 activity is essential in initiating hypoxia PH at a very early stage (4 days).

Normal pulmonary artery endothelial cells display unique responses to acute hypoxia in a Hif2α dependent manner

Our short-term in vivo hypoxia studies support the hypothesis that HIF2 activity is activated by hypoxia very early in one or multiple pulmonary vascular cells (EC, SMC, and Fibs), initiating PH development. Here, we attempted to determine the main cell type(s) whose activation by acute hypoxia may explain the observed events in 4 day hypoxia rats (Fig 5) by first comparing and contrasting the response of normal human pulmonary artery derived EC, SMC, and Fibs to acute hypoxia. We chose to use normal vascular cells, as we were studying the function of HIF in PH initiation. Acute hypoxia (1.5% for 16 hours) significantly activated expression of several classic HIF target genes including Adm, Ca9, Glut1, Ndrg1, and Vegfa in all three cell types, suggesting activation of these classical HIF target genes lacks cell-type specificity (Fig 6A). Hypoxic induction of the pro-inflammatory genes Sdf1, Cxcr4, and Icam1 was most prominent in ECs (Fig 6B). Additional unique responses of ECs to acute hypoxia was evidenced by reduced expression of Id1 and Id3, and increased expression of the growth factor Tgfa, a ligand of Egfr (Fig 6C), all of which can promote cell proliferation and survival. However, induction of Apln, Edn1, Pdgfb, and Tsp1 was mainly observed in SMC and Fibs while Arg1 and Arg2 were not induced by acute hypoxia in any of the three cell types (Fig. 6C). These data support a unique role of EC in response to acute hypoxia, to increase genes involved in inflammation and cell migration and proliferation. Thus, we

determined if HIF2 is responsible for increased expression of inflammatory and proliferative genes in EC under hypoxia. Interestingly, HIF2 inhibitor PT2567 completely abolished hypoxia-mediated changes of inflammatory (Cxcr4, Icam1 and Sdf1) (Fig 7B) and signaling/proliferation (Id1, Id3 and Tgfa) (Fig 7C) genes in control ECs, but only attenuated hypoxic induction of classical HIF target genes (Fig 7A). The essential role of HIF2, but not HIF1 in regulating inflammatory and signaling/proliferation genes in control ECs was further confirmed using an siRNA approach as HIF2a siRNA, but not HIF1a siRNA significantly attenuated changes of inflammatory and signaling/proliferation gene expression, induced by hypoxia in ECs (Suppl Fig 5C and D). While the direct function of proteins such as ID1 and ID3 is likely intracellular, the increased production of genes/proteins such as SDF1 and TGFA in EC could also involve paracrine signaling. To assess the role of activated ECs in regulating other pulmonary vascular cells, we prepared conditional-medium from normal ECs (EC-CM) cultured under normoxia or hypoxia, in the presence or absence of a HIF2 inhibitor PT2567. We found that HIF2-mediated activation of control EC also increased the expression of genes involved in cell proliferation (CCNE1 and CCNE2), pro-inflammation (CCL2), and anti-apoptosis (BCL2, BCL2L1, and BIRC5) in control SMC. This suggests a role for HIF2-signaling in activating EC, which promote SMC activation (Suppl. Fig 6). Thus, our studies support a hypothesis that activation of EC by hypoxia in a HIF2 dependent manner (but not HIF1), activates EC, also initiating proliferation of other vascular cells (SMC) and recruitment of monocytes/macrophages via increased expression of inflammatory cytokines (SDF1) and growth factors (Tgfa).

Hif2α expression in endothelial cells is required for hypoxia-induced PH and vascular remodeling in mice

To confirm the essential role of EC Hif2α in PH development in vivo, and to better understand the role of EC HIF2 in PH development, we generated and exposed EC Hif2α knockouts and their controls to normoxia or hypoxia for 5 weeks. As expected, Hif2α$^{fl/fl}$-EC-Cre$^-$ animals subjected to hypoxia displayed an increase in hematocrit (Fig 8A) and PH development (Fig 8B-D). We observed a similar increase of hematocrit in the hypoxia-exposed Hif2α$^{fl/fl}$-EC-Cre$^+$ mice, confirming previous reports that endothelial Hif2α is not required for hypoxia-induced erythropoiesis and erythrocytosis (4). However, hypoxia exposed Hif2α$^{fl/fl}$-EC-Cre$^+$ mice exhibited no signs of PH (Fig 8B-D). The increase in the number of αSMA-positive pulmonary vessels in hypoxia-exposure HIF2α$^{fl/fl}$-EC-Cre$^-$ mice was also abolished in hypoxia-exposure Hif2α$^{fl/fl}$-EC-Cre$^+$ mice (Fig 8E-F). We also examined the same set of genes that we studied in rats treated with the HIF2 inhibitor (Suppl Fig 3). As expected, genes induced by hypoxia in the lungs of control rats (Suppl Fig 3) were partially overlapping with the genes induced in mice by chronic hypoxia (Suppl Fig 7). The list included Ndrg1, Sdf1, Arg1, Edn1. However, the only genes that were reduced in both hypoxia exposed rats treated with HIF2 inhibitor (Suppl Fig 3) and in mice with EC Hif2α KO were Sdf1, Arg1, and Edn1 (Suppl Fig 7). This indicates the particular importance of these genes in the development of hypoxia-induced PH. Interestingly, we found that Acta1 and Myh7 gene expression was markedly reduced in the RV of hypoxic Hif2α$^{fl/fl}$-EC-Cre$^+$ mice (Suppl Fig 8), compared to RV of hypoxic Hif2α$^{fl/fl}$-EC-Cre$^-$ mice, confirming reduced RV remodeling.

DISCUSSION

Using both genetic and pharmacological approaches to inhibit global HIF1 or HIF2 activity, we found that inhibition of HIF2α, but not HIF1α, attenuates the development of PH in normal adult animals exposed to chronic (4-5 weeks) hypoxia. Importantly, in addition to addressing the role of HIF1 and HIF2 in hypoxia-induced PH development using normal adult animals, we also uncovered several novel roles of HIF2 in adult animals including its requirement for animals to survive under chronic hypoxic conditions. Our in vitro studies (pulmonary vascular cell's response to acute hypoxia), in combination with short-term hypoxia in vivo studies, support a hypothesis that EC HIF2 is "essential" in PH initiation because, only ECs can increase the production of diffusible cytokines (SDF1), that may recruit monocytes/macrophages and other blood/bone marrow derived cells to lung vessels, at early stages of PH development. This result is also consistent with an earlier study showing that EC-derived Sdf1 contributes to PH in PHD2-deficient mice (3). Our studies also support the hypothesis that HIF2 in SMC or Fibs is "not essential" in PH initiation. This might be due to the fact that more than one cell type (among SMC, Fibs, and EC) can induce the expression of the PH-related genes such as Apln, Edn1, Pdgfb, Tgfa, and Tsp1. Thus, our studies contribute to a better understanding of the role of HIF2 in PH initiation.

PH is observed in post-natal humans and animals with normal pulmonary circulations in response to chronic hypoxia stress. However, to study the role of HIF in PH development, most published studies used models in which HIF deletion or HIF activation (in PHD2 knockout) was initiated in embryonic life (1, 3-8, 10). We think results from such approaches needed to be re-evaluated because 1) all these models

are known to exhibit vascular defects in developing embryos and likely in adult mice derived from these embryos (12-17) and 2) there are examples demonstrating differences in phenotype (baseline as well as stressed) when gene deletion is initiated in the embryo versus in the adult (27-29). For example, inhibition of monocyte chemoattractant protein 1 (MCP1) initiated in adult animals reduces hypoxia- or MCT-induced PH (27, 28). In contrast, MCP-1 or MCP-1 receptor knock-out mice (initiated in the embryo) exhibit spontaneous PH (29). Our data showing the effects of HIF2 inhibition in normal adult animals demonstrates the functional importance of HIF2 in PH development, eliminating the concerns that HIF2's function in most previous reported studies is due to the defects in the lung vasculature and heart by Hif2α-deletion or activation in developing embryos. Although we also used mice with constitutive EC-specific knockout of Hif2α, initiated in the embryo, we recognize that results from the EC HIF2a KO study have limitations. However, the purpose of our EC HIF2a knockout study is not to determine the general function of HIF2 in hypoxia-induced PH, but to provide a molecular explanation for the function of HIF2 in EC in PH development. Results from *in vivo* studies in these mice confirmed a) results obtained from mice with an inducible, global knockout, b) results from animals treated two different inhibitors and c) results from *in vitro* studies.

The role of Hif1α in the development of hypoxia-induced PH is controversial: Studies demonstrated either a partial amelioration of PH (10, 11), or a temporary slowing of PH progression (1) or even elevated PAPs (2), in mice with reduced HIF1a expression. Additional in vivo studies in which HIF1 is activated by genetic manipulation of PHDs or the VHL protein, demonstrated Hif1α was not required for PH development

(3, 4, 9). We speculate that the different results reported could be due to using hypoxia or pseudo-hypoxia (PHD or VHL knockout animals) approaches or deletion of Hif1a gene in different cell types or deletion of Hif1a initiated in embroys or adult mice. Our studies using global (not cell-type specific) inhibition of HIF1a in normal adult animals (not initiated in embryo) and using hypoxia to activate HIF (not pseudo-hypoxia), do not support an indespensible role of Hif1α for PH in animals after 4-5 weeks of hypoxia exposure. However, we cannot exclude a transient role of Hif1α in the earlier stage of PH development, which we did not investigate. Indeed, a transient role of Hif1α in hypoxia-induced PH has been reported (1). Also, we cannot exclude disease-relevant Hif1α-signaling in SMC *in vivo*, as we did not confirm sufficient knockdown in this cell type in our ASO and our inducible knockout system. Last, our *in vivo* studies were performed using hypobaric hypoxia, while previous studies were performed using normobaric hypoxia. Since some physiological responses differ depending on the mode of hypoxia (30), the different experimental setting might also contribute to some of the differences between our findings and previous studies on the role of Hif1α in hypoxic PH.

Another distinct feature of this study is to inhibit global, not cell type specific HIF activity for purpose of determining HIF function in PH development. Cell-type specific knockout is a powerful method to understand the contribution of the targeted gene in a specific cell type to the disease. However, we believe conclusions derived solely from cell-type specific knockout experiments may have shortcomings for the purpose of determining the general function of a gene in a disease such as PH where interactions between multiple cell types are clearly necessary for disease progression and a specific

gene may have different or opposite functions in different disease-involved cell types. For example, caveolin-1 is reduced in PH EC and its reduction in EC promotes PH development (31); however, caveolin-1 is over-expressed in PH SMC and its over-expression in SMC also promotes PH development (31, 32). Using global HIF gene deletion or pharmacological inhibition, we concluded that HIF2, not HIF1 is important for hypoxia-induced PH development. However, EC Hif2a gene deletion (Fig 8) appears to be more effective than global HIF2 reduction (ASO and HIF2 inhibitor PT2567) in preventing development of hypoxia-induced PH, suggesting the true role of HIF2 in hypoxia-induced PH development is less important than what are reported in EC HIF2a deletion models.

Cell type specific knockout approaches may also miss the possible side effects of therapeutic inhibition of a gene such as Hif2α, especially if Hif2α also plays important roles in other cell types for other processes. Indeed, complete global deletion of Hif2α is detrimental for mice's survival under chronic hypoxia, which is a novel and important finding. Although the underlying causes for this lethality and the different tolerance to chronic hypoxia between Hif2α KO mice and mice/rats treated with Hif2α inhibitor are beyond the scope of this study, we speculate that mice with significant loss of Hif2α might have succumbed to cardiogenic shock under hypoxic conditions. This is based on our findings that Hif2α-ASO-treatment reduced levels of circulating catecholamines that were significantly increased in hypoxia in control-ASO mice (Suppl. Fig. 2B). Hif2a-ASO also abolished the increase in heart rate that was observed in control mice with hypoxia-exposure (Suppl. Fig. 2C), resulting in reduced cardiac output (Suppl. Fig. 2D). Hif2α knockdown also resulted in lower dP/dt$_{max}$ (RV), a parameter of ventricular systolic

function (Suppl. Fig. 2E). These effects could be mediated by loss of Hif2α in heart tissue (Suppl. Fig. 2A) or by loss in other organs (e.g., the adrenal glands). In conclusion, we speculate that lower hematocrits in Hif2α-ASO-treated animals in addition to the observed changes in cardiac function, could result in critically low delivery of oxygen (DO_2) and finally death of Hif2α KO mice. This interpretation of our data is consistent with previous studies, demonstrating Hif2α-dependent changes in catecholamines (8), heart rate and cardiac output and physiology (33-35). Importantly, we did not observe changes in heart rate in mice with EC-specific Hif2α knockout, demonstrating that the observed reduction in RVSPs in Hif2α-ASO-treated animals was not merely due to impaired cardiac function. In summary, our study suggests that we need to exercise caution, particularly for patients residing at high altitude, if a Hif2α inhibitor is going to be used in clinic in future.

Most published studies have evaluated the end effect of HIF inhibition in animals exposed to chronic hypoxia, without knowledge of the role of HIF in early stage of PH initiation. We performed studies in animals exposed to chronic as well as short-term hypoxia. We revealed that HIF2 is essential in several early events (macrophage recruitment and vascular cell proliferation) of PH development, likely by activating genes such as Sdf1 (inflammation) and Tgfa (cell proliferation) in vivo.

To our knowledge, this is the first time that the responses of the three primary normal human pulmonary vascular cell types (EC, SMC and Fibs) to acute hypoxia have been examined concurrently for a set of genes that have been reported (3, 4, 6, 9, 22-24) and demonstrated in our current studies (Suppl. Figs 3, 4 and 7), to be important for PH development. Our studies lead to novel findings that EC is the primary cell type

that can be activated by short-term hypoxia (Id reduction), in a HIF2 dependent manner to produce inflammatory (Sdf1) and growth promoting factors (Tgfa) (Fig 7), to activate other vascular cells (Suppl Fig. 6). Our findings from short-term in vivo and in vitro studies are consistent with previous studies by us and others that recruitment of bone-marrow/blood derived cells such as monocytes/macrophages is an early and critical event in PH development (25, 36-39) and cytokines/chemokines including Sdf1 are among the earliest inflammatory factors increased in the pulmonary arteries of hypoxia-exposed animals and whose increase precedes monocyte/macrophage accumulation (26). Our in vitro studies also indicated that normal SMC and Fibs can be acutely activated by hypoxia to produce factors such as Apln, Edn1, and Tsp1 that have been shown to be involved in vessel constriction (4, 6). While knockout of Hif2α in SMC does not prevent hypoxic PH (7), studies on the effect of Hif2α inhibition in Fibs, in combination of in other pulmonary cell types, such as SMC, are needed. Further, our data indicated that there is no hypoxic induction of Arg1 and Arg2 in normal EC, SMC and Fibs (Fig 6) although we consistently observed increased expression of Arg1 and Arg2 in lungs of chronic and short-term hypoxia exposed rodents and whose expression is reduced by HIF2 inhibition (Suppl Figs 3, 4, 7). These data suggested that increased expression of genes such as Arg1 and Arg2 could be mainly from recruited cells.

The critical role of HIF2 in the development of hypoxia-induced PH has raised significant interest in targeting HIF2 for treatment of PH patients. In fact, one study has already demonstrated a quite effective role of a HIF2 inhibitor in reversing PH in animal models (40). However, small animal models for PH have well-recognized limitations (41). More importantly, targets that have been demonstrated to be effective in animal

models are often failed in clinical trials (42, 43). Thus, we believe further studies are needed before initiation of a HIF2 inhibitor clinical trial in humans. Accumulating data indicates that vascular cells established from PH patients and large animals (cows) exhibit and maintain their unique phenotypes in vitro (44). We believe these cells could provide an excellent platform to further determine the role of HIF2 for PH treatment. In addition, although Hif1 is not essential in hypoxia-induced PH development, the role of Hif1 in PH disease maintanace is also possible.

In summary, our studies have demonstrated a positive role of HIF2 in PH development in response to chronic hypoxia in normal adult animals. However, more research is needed to determine if HIF2 is truly a good PH treatment target because HIF2 activity is likely to be one of the many factors that are required, acting in different cell types, at different stages, to initiate PH (45). In addition, there are data from cancer research that factors/pathways that initiate cancer often fail to be good treatment tragets because there are many additional changes occur during cancer progression.

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Figure 1: *Global Hif1α is dispensable in establishing hypoxia-induced PH at 5 weeks in adult mice.* **A)** Experimental setup: Tamoxifen was injected into mice daily during week 1 to activate Cre and delete Hif1α gene, then all mice were moved to sea levels for one week, followed by exposing mice to either normoxia (P_B-740mmHg) or hypoxia (simulated altitude of 18,000ft, P_B=370 mmHg) for 5 weeks. After five weeks hypoxia exposure, readout parameters were measured and animals were euthanized. **B)** Hematocrit (Hct) levels. **C)** Right-ventricular systolic pressure (RVSP). **D)** Fulton index (ratio of weight of the RV to the weight of the LV and septum) and **E)** Ratio of RV-weight to bodyweight. Animal number in each group, under normoxia or hypoxia can be found in Fulton Index panel (Fig 1D). Due to technical difficulties, we were not able to obtain readings of Hct and/or RVSP for some mice, thus the animal number for these data was typically less than the animal number for Fulton index in this and other Figures of this study. * is for difference between hypoxia versus normoxia in the same genotype (or treatment) group in this and all other figures in the paper. Statistical analysis showed here is 2-way ANOVA analysis.

Figure 2: *Global Hif2α gene deletion is incompatible for mouse survival under hypoxic conditions while global partial Hif2α gene deletion diminishes hypoxia-induced PH development at 5 weeks in adult mice*. Mice were treated with tamoxifen and exposed to normoxia or hypoxia as described in Fig 1A. **A)** Kaplan-Meier-Curve for survival of *Hif2α^{fl/fl};UBC-CreERT^+* mice during hypoxia exposure, compared to survival of *Hif1α^{fl/fl};UBC-CreERT^+* and *Hif2α^{fl/fl};UBC-CreERT^-* mice during exposure to hypoxia. More than 9 mice in each group were used for this experiment. B-E: Hemodynamics of Hif2α^{fl/WT};UbcCreERT^+ and Hif2α^{fl/WT};UbcCreER^- mice after 5 week exposure to normoxia or hypoxia*. **B)** Hematocrit (Hct) levels. **C)** RVSP. **D)** Fulton index. **E)** ratio of RV-weight to bodyweight. # is used to show the differences between genotypes or treatments under hypoxic condition in this and all other figures of this study. Statistical analysis showed here is 2-way ANOVA analysis (note in panel C there is a significant decrease in RVSP at 18,000 in UbcCre+ animals analyzed by unpaired T-test p<0.05).

Figure 3: *Knockdown of Hif2α but not Hif1α utilizing antisense-oligonucleotides significantly reduces development of hypoxia-induced PH at 5 weeks in adult mice.* **A and B)** Testing the effectiveness and specificity of antisense-oligonucleotide in a pilot experiment. Wild-type C57bl/6J mice were treated either with injections of an antisense-oligonucleotide targeting Hif2α mRNA (Hif2α-ASO) or equal volumes of 0.9% NaCl (Ctrl) at days 1, 4, 8 and 11. At day 12, mice (N=3) were sacrificed and multiple organs were collected for RNA preparation. **A)** Levels of Hif1α and of Hif2α mRNA were quantified by qRT-PCR in indicated organs. **B)** Levels of *Pgk1* (a HIF1 target gene) and *Epo* (a HIF2 target gene) in kidneys from mice targeted with NaCL or Hif2α-ASO, were quantified by qRT-PCR. **C)** Experimental setup. In week 2, mice were kept under sea level and began to receiving injection of Control, Hif1α-ASO, or Hif2α-ASO (two injections per week at Monday and Thursday). Starting week three, mice were exposed to either sea level or 18,000ft for 5 weeks, in which two injections per week were maintained. D-K) Endpoint measurements for the experimental animals in C. **D)** Hct. **E)** RVSP. **F)** Fulton index. **G)** ratio of RV-weight to bodyweight. **H-J)** alpha-SM-actin positive pulmonary vessels. **K)** Summary of Hct, RVSP, Fulton index, ratio of RV-weight to bodyweight for mice targeted with control or Hif1α-ASO under normoxia or hypoxia. Statistical significance as determined by t-test (A, B, J, and K) or 2-way ANOVA (D-G). ^ is used to show the differences between genotypes or treatments under normoxic condition in this and all other figures of this study.

Figure 4: *Small molecule Hif2 inhibitor PT2567 significantly attenuates development of hypoxia-induced PH at 4 weeks in adult rats.* Sprague Dawley (SD) male rats weighing 210-245 grams (Charles

River Laboratories) were housed in chambers under normoxia or hypoxic (high altitude ~ 18,000 feet) conditions for four weeks. Rats were dosed with vehicle methylcellulose (0.5%)/Tween-80 (0.5%) or PT2567 (300 mg/kg/day), beginning the day they were placed in chambers. After 4 weeks, endpoint measurements for the experimental animals were conducted (A-C). **A**) Mean Pulmonary Arterial Pressure. **B**) Ratio of weight of right ventricle versus weight of left ventricle. **C**) Representative images of pulmonary vessels stained with anti-macrophages/monocytes antibody, clone ED-1 (Top), anti-Ki67 antibody (Middle), or anti-Tenascin C antibody (Bottom). The levels of CD68, CCNA1, and TNC mRNAs in the whole lung tissues of indicated rats were also shown (Right). **D, E**) α-SM-actin positive pulmonary vessels, scale bar: 100μm. Statistical significance determined by 2-way ANOVA (A, B) or by t-test (C-E).

Figure 5: *Small molecule Hif2* inhibitor *PT2567 significantly attenuates early events in hypoxia-exposed adult rats at 4 days.* Sprague Dawley rats were housed in chambers under normoxia or hypoxic conditions for four days. Rats were dosed with vehicle or PT2567 as described in Figure 4. After 4 days, endpoint measurements for the experimental animals were conducted (A-C). **A**) Mean Pulmonary Arterial Pressure. **B**) Ratio of weight of right ventricle versus weight of left ventricle. **C**) Representative images of pulmonary vessels stained with anti-macrophages/monocytes antibody, clone ED-1 (Top), anti-Ki67 antibody (Middle), or anti-Tenascin C antibody (Bottom). The levels of CD68, CCNA1, and TNC mRNAs in lungs of indicated rats were also shown (right). Statistical significance determined by 2-way ANOVA (A, B) or by t-test (C).

Figure 6. Normal human pulmonary artery vascular cells exhibit unique properties in response to acute hypoxia. Normal human pulmonary artery vascular cells (EC, Fibs, SMCs, N=5 for each cell type) were cultured under normoxia or hypoxia (1.5% O2) for 16 hours, and then cells were collected for RNA preparation. The same set of genes that were examined in vivo were studied here. Results were from at least 5 different cell populations for each cell type, in which result for a specific cell population was from three independent N or H experiments here or other similar experiments in this paper. **A**) Classical HIF target genes. **B**) Genes involved in inflammation. **C**) Genes involved in signaling. Statistical significance determined by t-test.

Figure 7. HIF2 inhibitor PT2567 significantly attenuates altered production of genes involved in inflammation and signaling in normal pulmonary endothelial cells in response to acute hypoxia. To determine if HIF2 activity is responsible for hypoxia-mediated gene expression changes in EC, normal human pulmonary artery EC cells (N=3) were cultured under normoxia or hypoxia (1.5% O2) for 16 hours, in the presence of DMSO (control) or different concentration of HIF2 inhibitor PT2567, and then cells were collected for RNA preparation and qRT-PCR. **A**) Select classical HIF target genes. **B**) Genes involved in inflammation that are significantly induced by hypoxia in EC (Fig 6B). **C**) Genes involved in signaling that are significantly altered by hypoxia in EC (Fig 6C). Statistical significance determined by t-test.

Figure 8. Hif2α expression in endothelial cells is required for development of hypoxia-induced PH. Endothelial Hif2α knockout or Hif2α WT mice were exposed to either sea level atmosphere or to simulated 18,000ft of altitude for 5 weeks. A-G are the endpoint measurements in these mice. **A**) Hct. **B**) RVSP. **C**) Fulton index. **D**) ratio of RV-weight to bodyweight; **E-F**) α-SM-actin positive pulmonary vessels. Statistical significance determined by 2-way ANOVA (A-D) or t-test (F).
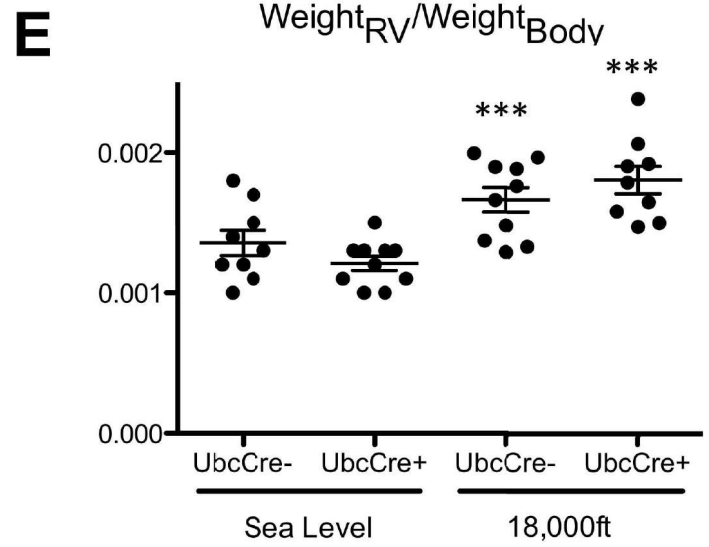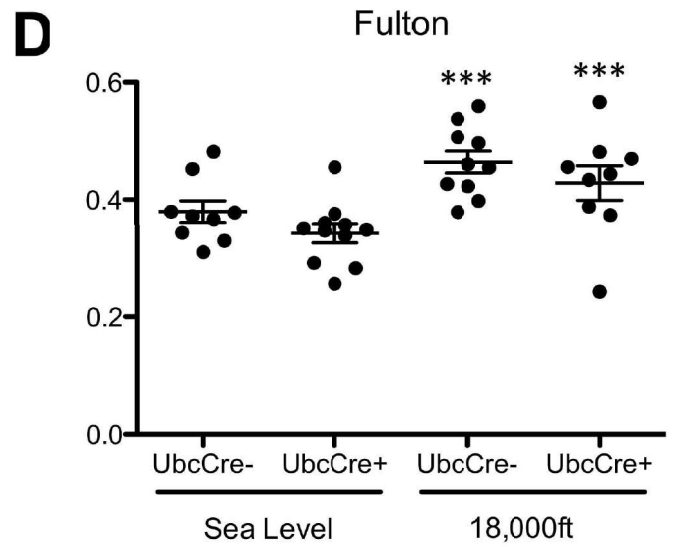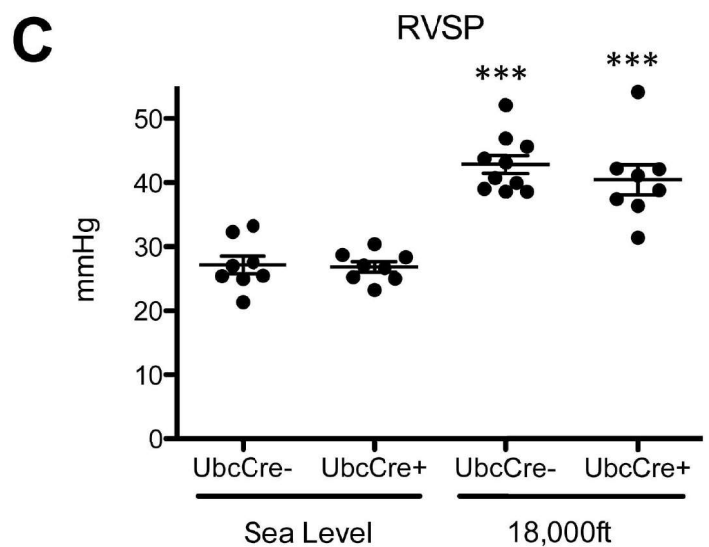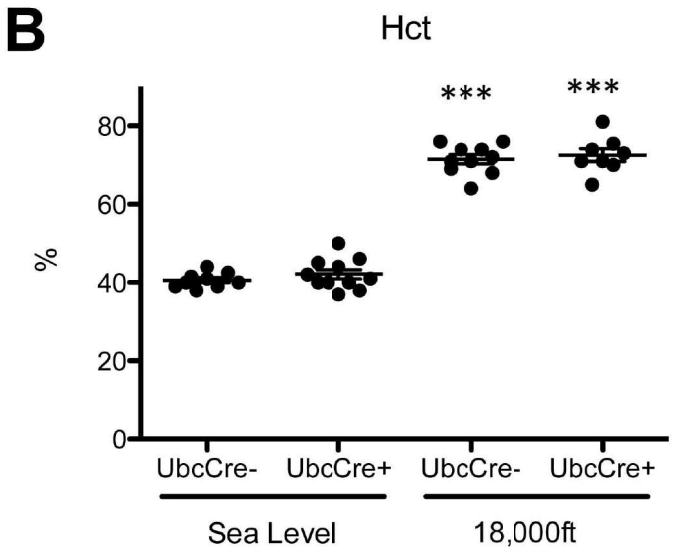
Figure 1

Figure 2

A

Kaplan-Meier Curve

Legend:
- UbcCre-
- Hif1a$^{fl/fl}$-UbcCre+
- Hif2a$^{fl/fl}$-UbcCre+

p<0.0001

Y-axis: Percent survival
X-axis: Time (days at +18,000ft)

B

Hct

*** ***

Y-axis: %

X-axis: UbcCre- UbcCre+ (Sea Level) | UbcCre- UbcCre+ (18,000ft)

C

RVSP

*** ***

Y-axis: mmHg

X-axis: UbcCre- UbcCre+ (Sea Level) | UbcCre- UbcCre+ (18,000ft)

D

Fulton

***
***
##

Y-axis values: 0.4, 0.2, 0.0

X-axis: UbcCre- UbcCre+ (Sea Level) | UbcCre- UbcCre+ (18,000ft)

E

Weight$_{RV}$/Weight$_{Body}$

***
***
##

Y-axis values: 0.0025, 0.0020, 0.0015, 0.0010, 0.0005, 0.0000

X-axis: UbcCre- UbcCre+ (Sea Level) | UbcCre- UbcCre+ (18,000ft)

Figure 3

Figure 3

H Sea Level

	Ctrl-ASO	Hif2a-ASO
10x		
20x		

I 18,000ft

	Ctrl-ASO	Hif2a-ASO
10x		
20x		

J

αSMA + vessels

K

	Ctrl-ASO		Hif1a-ASO		
	Mean	*+/- SEM*	*Mean*	*+/- SEM*	*p-value*
Hct (%)	66	0.5	67.9	2.17	0.1412
RVSP (mmHg)	42.62	2.17	43.08	1.60	0.8608
Fulton	0.3455	0.0185	0.3457	0.0173	0.9940
Weight$_{RV}$/Weight$_{Body}$	0.00112	0.00006	0.00110	0.00007	0.8656

Figure 4
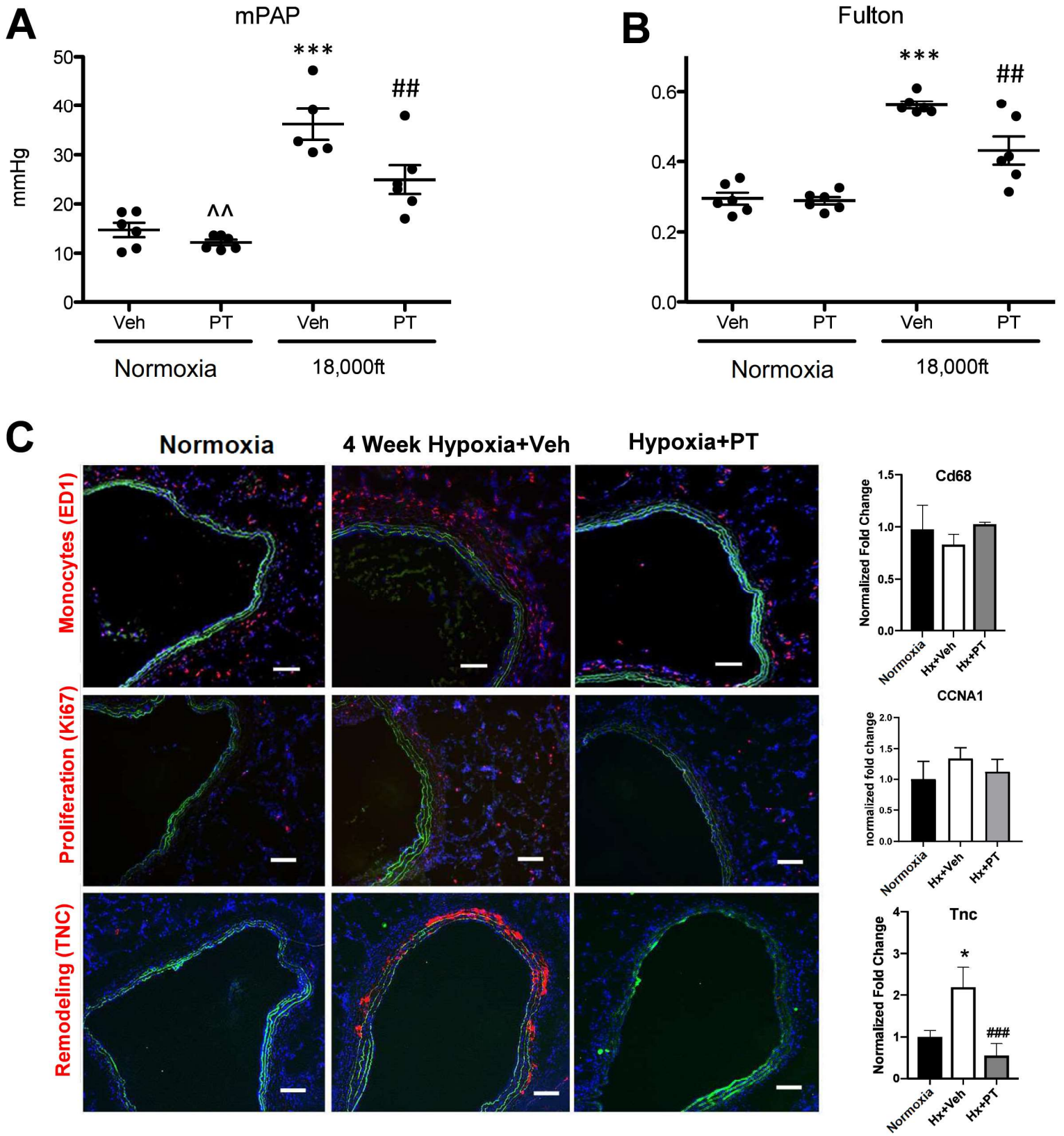
Figure 4

D
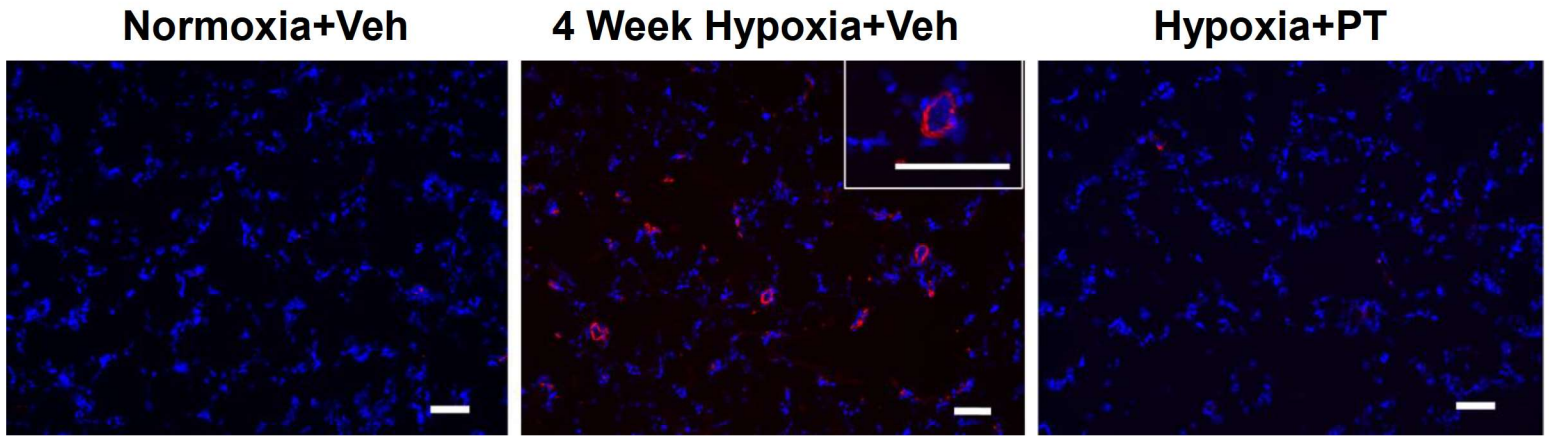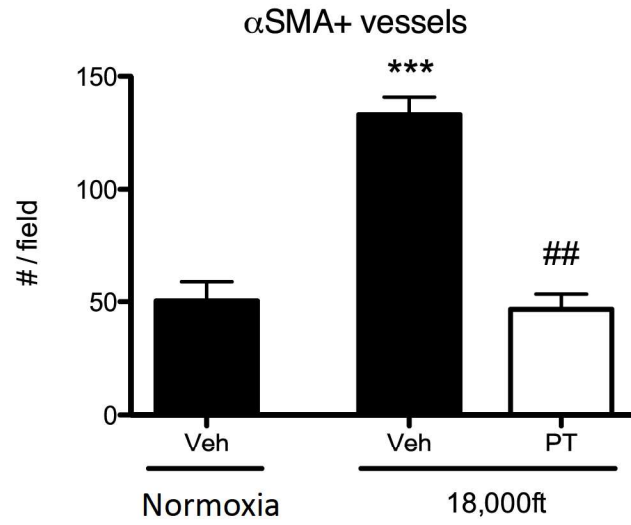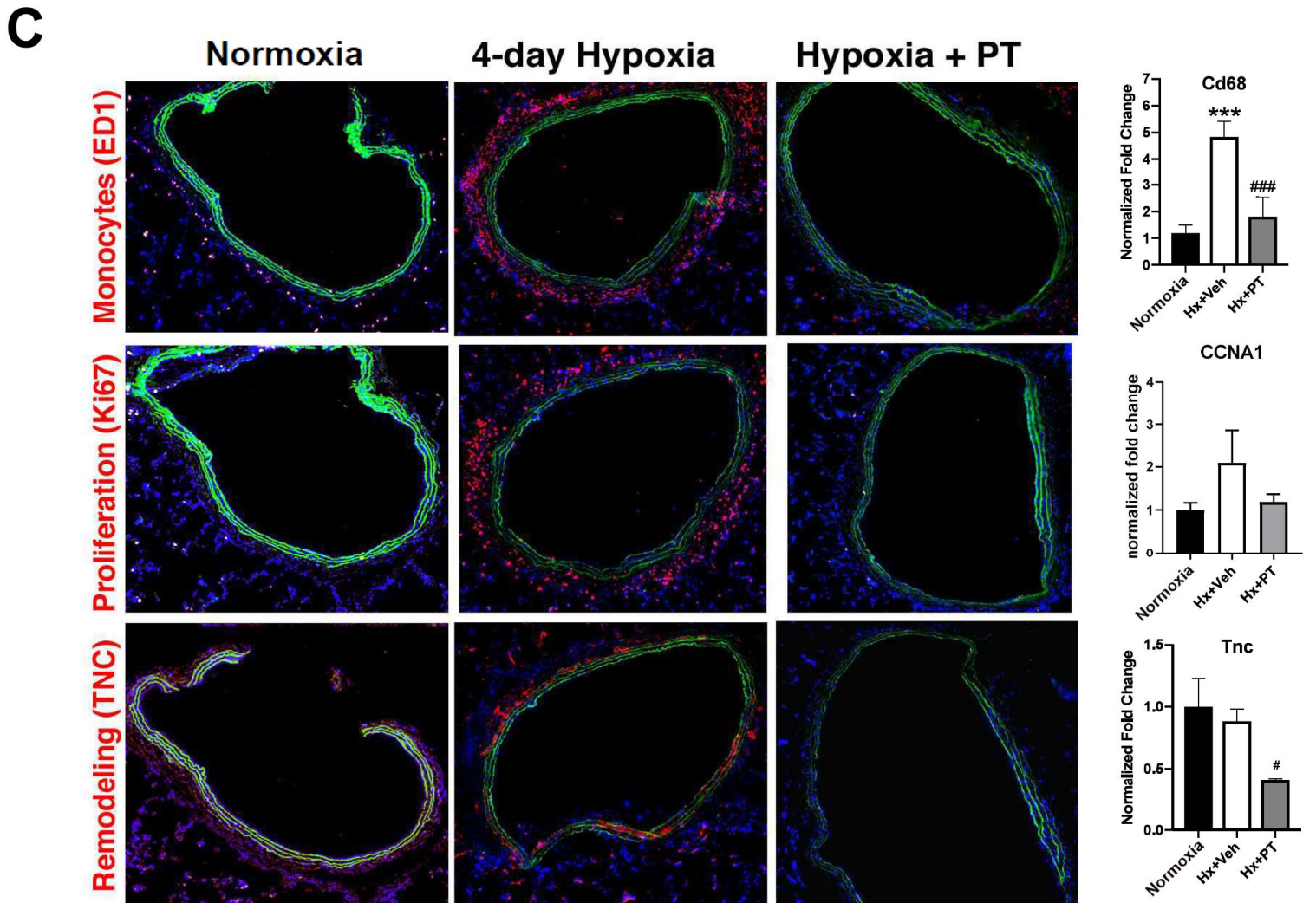
| Normoxia+Veh | 4 Week Hypoxia+Veh | Hypoxia+PT |

E

αSMA+ vessels

y-axis: # / field

150
100
50
0

*** (above 18,000ft Veh bar)
(above 18,000ft PT bar)

Veh | Veh | PT

Normoxia | 18,000ft

Figure 5

Figure 6

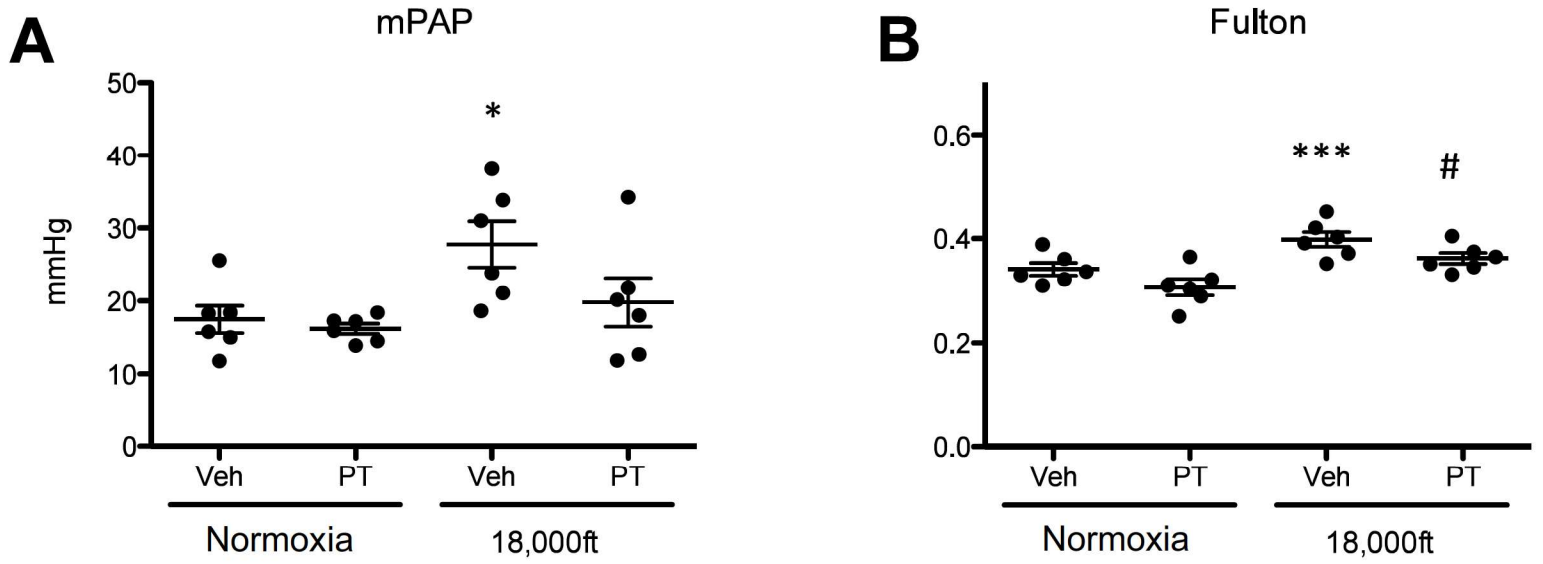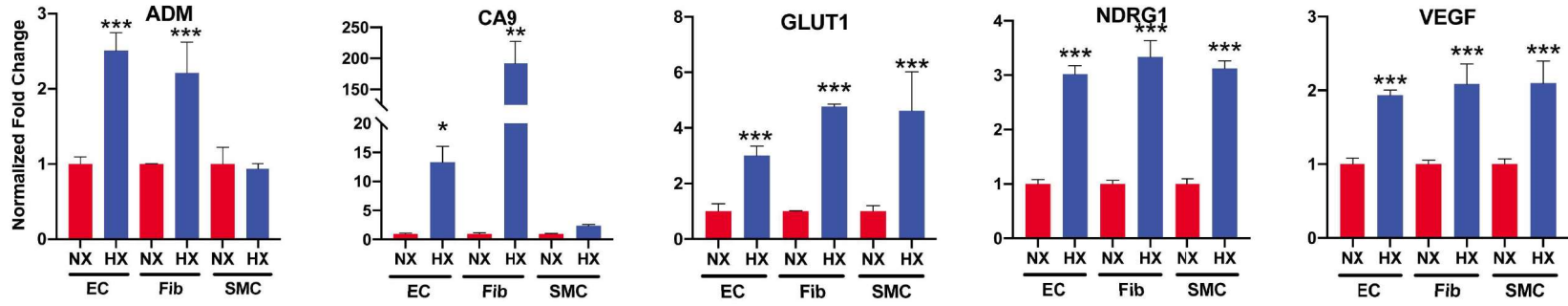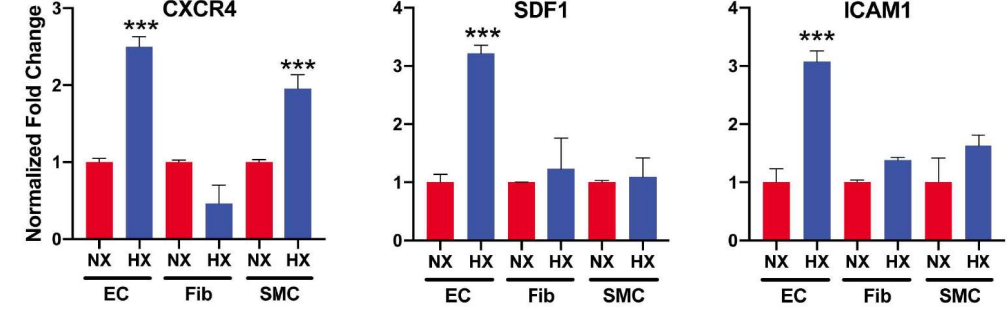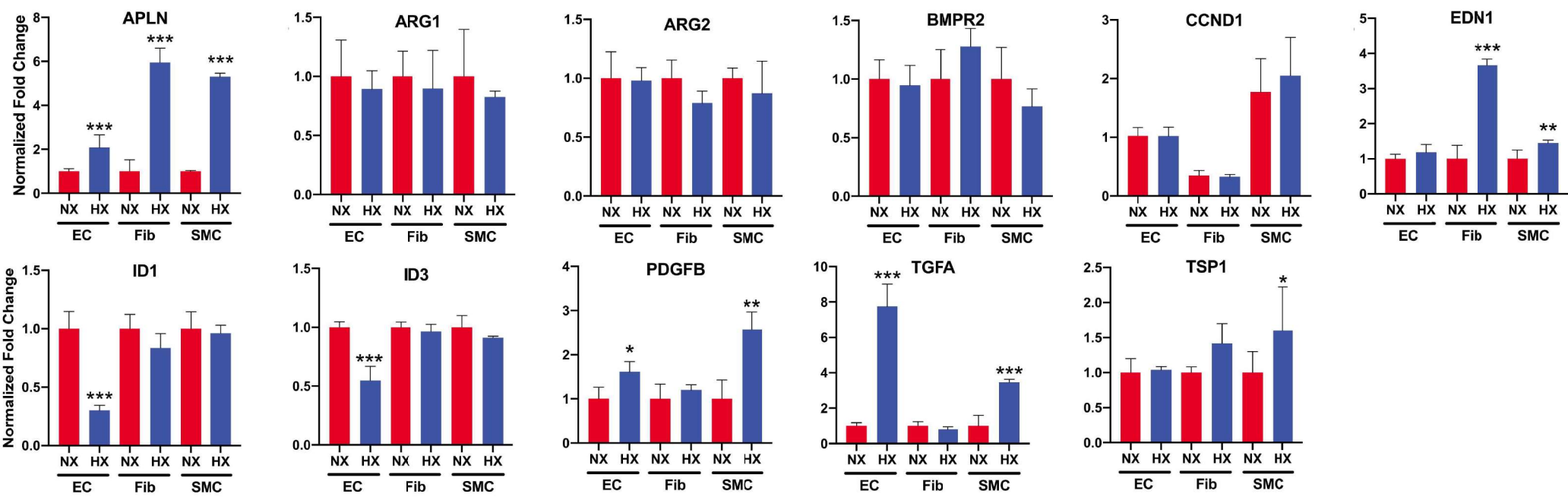**A** Hypoxic Induction of Classical HIF Target Genes in Normal Pulmonary Artery Vascular Cells
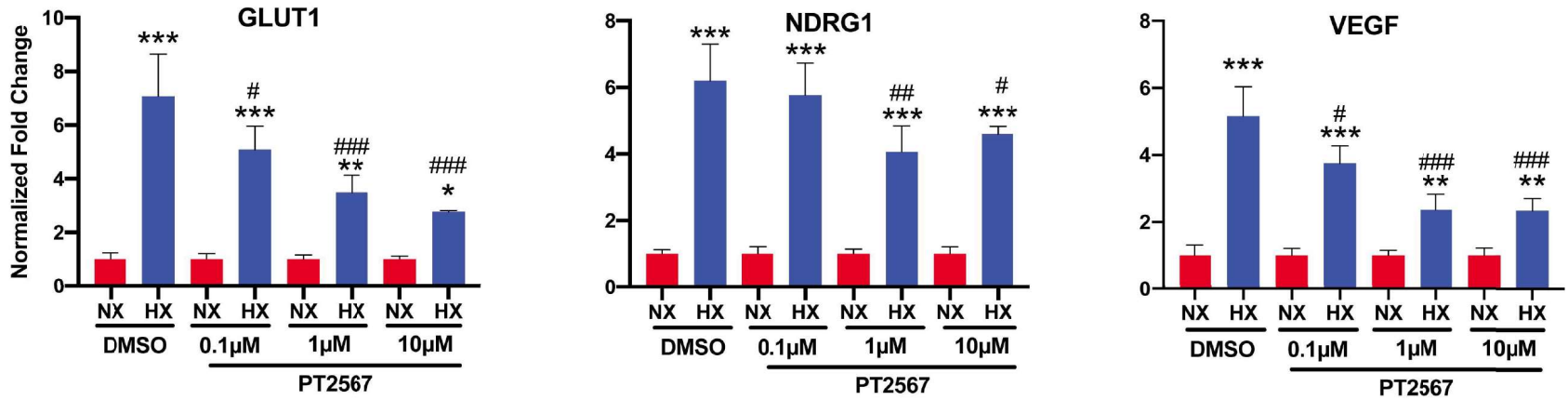
B Hypoxic Induction of Inflammatory genes in Normal Pulmonary Artery Vascular Cells
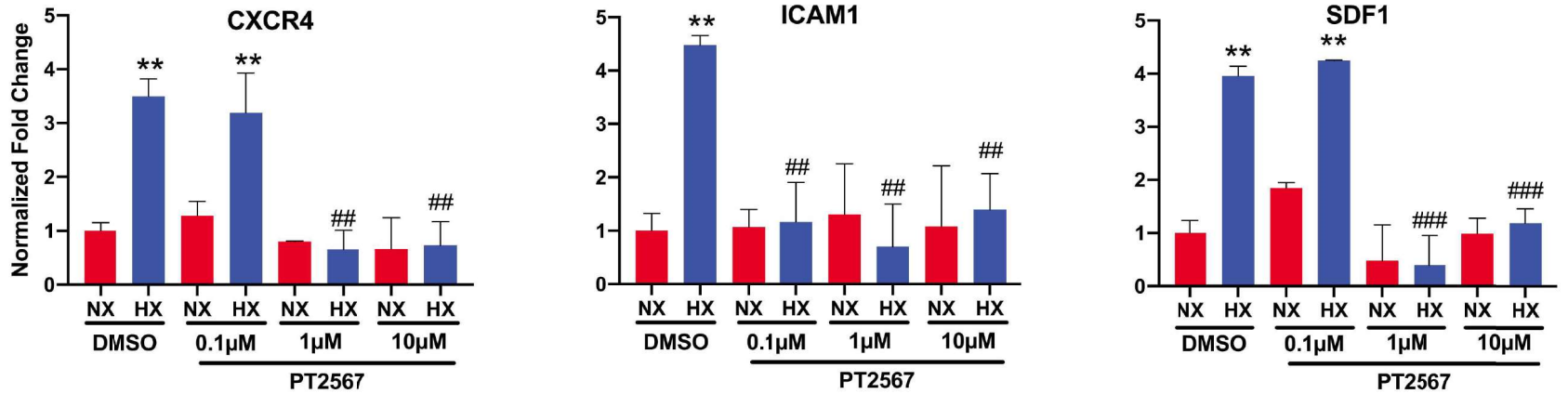
C Hypoxic Induction of Signaling Factors in Normal Pulmonary Artery Vascular Cells

Figure 7

A Hypoxia Induction of Classical HIF Target Genes in EC in Response to HIF2 Inhibitor

B Hypoxia Induction of Inflammatory Genes in EC in Response to HIF2 inhibitor

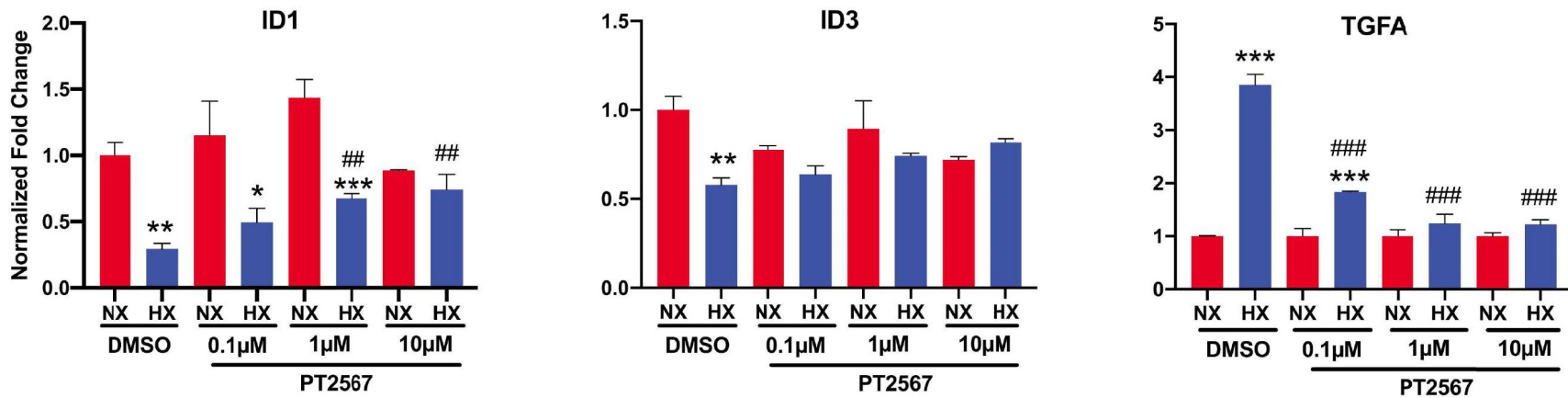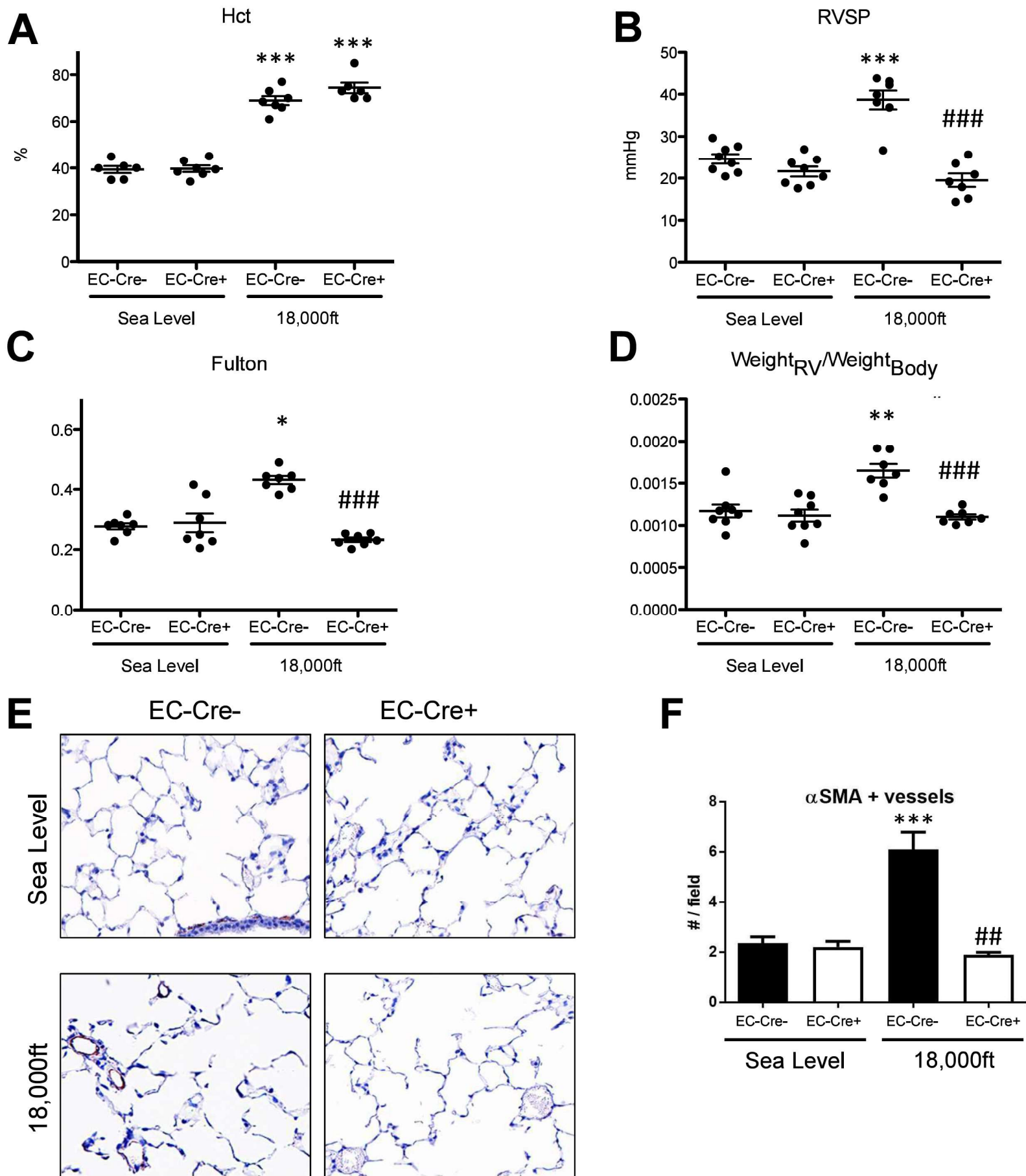**C** Hypoxia Induction of Signaling Factors in EC in Response to HIF2 Inhibitor

Figure 8

A Hct

B RVSP

C Fulton

D Weight$_{RV}$/Weight$_{Body}$

E EC-Cre-   EC-Cre+

F αSMA + vessels

Supplemental File

METHODS

Animals. All procedures with animals in this study were performed according to approved protocols under the supervision of the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus (Aurora, CO). All mice were C57bl6/J background. The experiments in mice began when they were 12 weeks old. Sprague Dawley (SD) male rats weighing 210-245 grams were used for rat experiments.

***Transgenic mice and genotyping**.*

Hif1α floxed mice (1), *Hif-2α* floxed mice (2) were constructed wherein exon 2 (coding DNA binding domain) of the Hif1α or Hif2α gene was floxed with LoxP sites. *UbcCreERT^{+}* mice express a fusion protein consisting of cre recombinase and the human estrogen receptor binding domain under control of a ubiquitously active Ubiquitin-C gene promoter, in which Cre recombinase is activated upon tamoxifen exposure. Crossing of Hif1α$^{fl/fl}$ or Hif2α$^{fl/fl}$ mice with UbcCreERT^{+} mice generated *Hif1α$^{fl/fl}$;UbcCreERT^{+}*mice, *Hif2α$^{fl/fl}$;UbcCreERT^{+}* mice and *Hif2α$^{fl/WT}$;UbcCreERT^{+}* mice, in which tamoxifen treatment completely or partially deleted *Hif1α* or *Hif2α* gene in all cell types in adult mice (See Suppl Fig 1A-C for breeding information and Suppl Table 1 for Hifa reduction efficiency). Endothelial cell–specific knockout of *Hif2α* was generated by crossing *Hif2α$^{fl/fl}$* mice to mice expressing cre recombinase under the control of the VE-cadherin promoter (from the Jackson laboratory, kindly provided by Dr. Eltzschig's laboratory, University of Colorado, Denver, CO, USA) (See Suppl Fig 1D for breeding

information)(3). In all cases, mouse genotypes were determined using real time PCR of tail genomic DNAs by a commercial vendor (Transnetyx, Cordova, TN, USA). Age-matched (about 12 wk old), male and female mice with the desired genotypes were used for experimentation.

Tamoxifen treatment in mice. To delete Hif1α or Hif2α gene in adult mice, *UbcCreERT⁺* or *UbcCreERT⁻* (as control) mice were injected with 100 µl of tamoxifen solution (in peanut oil at 10mg/ml) i. p. once daily over five consecutive days. Mice were then allowed to rest for one week before being exposed to hypobaric hypoxia or normoxia.

Antisense oligonucleotide treatment in mice. Hif1α antisense oligonucleotide (henceforth *Hif1α*-ASO; 75mg/kg; 298745-11) or *Hif2α-ASO* (50mg/kg; 589983-5) or non-specific ASO (control-ASO; 549148-18) was injected intraperitoneally twice per week, beginning one week before the hypoxia exposure and continued during the hypoxia exposure. ASOs were provided by Ionis Pharmaceuticals, Inc. (Carlsbad, CA, USA). See Suppl Table for HIF1α and HIF2α mRNA reduction rate.

Rats treated with HIF2 inhibitor PT2567. Sprague Dawley (SD) male rats weighing 210-245 grams (Charles River Laboratories) were housed in chambers under normoxia or hypoxic (high altitude ~ 18,000 feet) conditions for four days or four weeks. Rats were dosed with vehicle methylcellulose (0.5%)/Tween-80 (0.5%) or PT2567 (300 mg/kg/day) (4), beginning the day they were placed in chambers. PT2567 was provided by Peloton Therapeutics, Inc.

Exposure to chronic hypoxia. Mice or rats were housed for indicated times (5 weeks for mice, 4 days or 4 weeks for rats) in chambers equipped for continuous hypobaric

hypoxia (0.5 atm, equivalent to 5,486m/18,000 feet altitude and corresponding to FiO_2 0.1). Chambers were vented to room atmosphere weekly for cage maintenance (twice weekly in case of ASO administration). Control mice were housed in identical chambers at sea level atmosphere (1.0 atm, 0m altitude, FiO_2 0.21).

Measurement of right ventricular systolic pressure in mice and mean pulmonary arterial pressure in rats. RV pressure measurements were performed essentially as previously described (5). Briefly, mice were anesthetized with isoflurane. The RV was directly catheterized with a fluid-filled catheter interfaced with a pressure transducer connected to a dedicated computer. Trains of successive pressure waveforms were analyzed and averaged over three seconds to determine RV systolic pressure (RVSP). MPAP measurements were performed as described (6). Additional measurements were performed in ASO-experiments utilizing a 1.4 French Pressure Volume Conductance System SPR-839 (Millar Instruments, Houston, TX) inserted into the RV via a surgical transdiaphragmatic approach.

Tissue preparation, hematocrit measurement, Fulton index and weight ratios. Animals were deeply anesthetized. Blood samples were taken by left ventricular puncture and the hematocrit was measured using glass capillaries and a suitable centrifuge (IEC, model MB micro-capillary centrifuge). Lung vasculature was flushed with PBS through the RV after incision of the left atrium. For immunohistochemical analysis, left lungs were inflated with agarose in situ and fixed in paraformaldehyde (4%) after excision. Left lungs were then paraffin embedded and cut into 5 μm sections. Right lung lobes were excised, snap frozen in liquid nitrogen and stored at -80°C for RNA extraction to conduct pulmonary gene expression analysis. To determine right

ventricular hypertrophy, the atria were removed from the heart. Subsequently, the heart was dissected into sections of RV free wall and LV free wall including the septum (LV+S) or LV only. Heart tissue was patted dry, weights were taken and the Fulton index (Fulton index = $Weight_{RV}/Weight_{LV+S}$) or $Weight_{RV}/Weight_{LV}$ and the ratio of $weight_{RV}$/bodyweight were calculated. After taking weights, heart tissue was snap frozen in liquid nitrogen and stored at -80°C for RNA extraction to conduct right ventricle gene expression analysis.

Immunohistochemistry of lung tissue*. Vascular remodeling was quantified by immunohistochemical staining with specific antibody and hematoxylin counterstaining as previously described (5). Alpha-smooth muscle actin antibody (αSMA) (Abcam ab 66133 rabbit polyclonal antibody; dilution 1:400) was used to assess vessel muscularization. Using a Nikon microscope, twelve fields ($1\mu m^2$ each) per lung were inspected and muscularized vessels (αSMA+) were counted. Anti-ED1 antibody, anti-Ki67 antibody, or anti-Tenascin C antibody was used to assess monocyte/macrophage accumulation, cell proliferation, or vessel remodeling respectively.

Cell culture and treatments. Normal human pulmonary artery endothelial cells and smooth muscle cells were provided by Pulmonary Hypertensive Breakthrough Initiative. Human ECs were cultured in endothelial cell basal medium plus supplements (Cat#: CC-3202, Lonza Walkersville, MD USA). Human SMCs were cultured in smooth muscle cell basal medium plus supplements (Cat#: CC-3182, Lonza Walkersville, MD USA). Human pulmonary artery Fibs are from control donors undergoing lobectomy or pneumonectomy at Papworth Hospital, Cambridge, UK and were cultured in fibroblast growth medium (DMEM) (15-018-CV, Corning, VA USA) supplemented with fetal bovine

serum (FBS) (10%, Cat#: 100-106, Gemini, CA USA) and Penicillin-Streptomycin (P/S) (30-002-CI, Corning, VA USA), MEM Nonessential Amino Acids (25-025-CI, Corning, VA USA) and L-Glutamine (4mM, 25-005-CI, Corning, VA USA). When hypoxia treatment was needed, cells were placed in normoxia (Nx, 21% O2) or hypoxia (Hx, 1.5% O2) for 16 h for HIF target gene studies, cultured under regular 10% FBS medium. To assess the role of HIF1 or HIF2 in the increased expression of HIF target genes in hypoxic EC cells, ECs were treated with HIF2 inhibitor PT2567 at concentration of 0.1, 1. or 10 μM or targeted with siRNA against Hif2α or Hif1α mRNA.

Gene expression analysis. After tissue disruption and homogenization using a bead-mill system, RNA from organs such as lung and heart, or cells was isolated using RNeasy column kits (Qiagen). Synthesis of cDNA was performed using a commercially available kit according to the manufacturer's instructions (iScript Advanced cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). Quantification of messenger RNA levels was performed by reverse transcription quantitative PCR (RT–qPCR) using iQ Universal SYBR Green Supermix (Bio-Rad) and CFX384 Real Time System (Bio-rad). All primer sets for RT–qPCR were validated for their specificity and amplification efficiency (85% to 110%) using melt curve analysis, RT–qPCR product sequencing and standard dilution analysis. Primer sequences are listed in Suppl. Table 6. The qPCR results were analyzed using the ΔΔCT method using 18S ribosomal RNA and beta actin messenger RNA as reference genes and presented in relative to samples from controls. For Fig. 3A) and Suppl. Fig. 8), commercially TaqMan probes and reagents were used (Applied Biosystems, Grand Island, NY), according to the manufacturer's instructions. At least

three independent experiments were performed for all in vitro results presented in this paper.

Statistics. In general, data is reported as mean +/- SEM. Statistical differences were evaluated by 2-way ANOVA test as well as an unpaired, two-tailed t-test. Figure legends specified the statistical analysis used for the data in each panel. Star (*) is used to indicate the difference between hypoxia versus normoxia in the same genotype (or treatment) group. # is used to show the differences between genotypes or treatments under hypoxic condition while ^ is used to mark the differences between genotypes or treatments under normoxic condition. Survival curves were compared by log-rank test. $P<0.05$ (* or # or ^) was considered statistically significant, other levels of statistical significance are reported as $p<0.01$ (** or ## or ^^) and $p<0.001$ (*** or ### or ^^^). Group size (n) is reported in the corresponding figure legends. Data was logged using Excel (Microsoft Corp.), graphing and calculations were performed using Graphpad Prism (GraphPad Software Inc.) and figures were designed using Powerpoint (Microsoft Corp.).

References

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Supplementary Figure 1. Breeding schemes to generate the transgenic mice used in the study. A) Breeding scheme to generate *Hif1α^{fl/fl}^;UBC-creER^{+/-}^ and Hif1α^{fl/fl}^;UBC-creER^{-/-}^* mice. **B)** Breeding scheme to generate *Hif2α^{fl/fl}^;UBC-creER^{+/-}^ and Hif2α^{fl/fl}^;UBC-creER^{-/-}^* mice. **C)** Breeding scheme to generate *Hif2α^{fl/wt}^;UBC-creER^{+/-}^ and Hif2α^{fl/wt}^;UBC-creER^{-/-}^* mice **D.)** Breeding scheme to generate *Hif2α^{fl/fl}^;Ve-cadherin-cre^{+/-}^ and Hif2α^{fl/fl}^;Ve-cadherin-cre^{-/-}^* mice.

Supplementary Figure 2. Hif2 ASO reduces cardiac function of mice under hypoxia. Mice were treated Control or HIF2a ASO as described in Fig. 3. At the end of the experiment, cardiac expression of Hif2a mRNA was determined by qRT-PCR (A). Levels of catecholamines in blood plasma as determined by HPLC (B). C-E: Parameters of cardiac function, Millar catheter measurements. Statistical significance determined by t-test (B) or by 2-way ANOVA (other panels).

Supplementary Figure 3. **HIF2 inhibitor PT2567 significantly normalizes the levels of some mis-expressed genes in lungs of rats exposed to hypoxia for 4 weeks**. A-C: levels of HIF target genes and PH related genes in the lungs from rats under normoxia or hypoxia, treated with control reagent or HIF2 inhibitor PT2567 (N=6 for each group). **A**) Classical HIF target genes; **B**) Genes involved in inflammation; **C**) Genes involved in signaling and proliferation. Statistical significance determined by t-test.

Supplementary Figure 4. **HIF inhibitor PT2567 significantly normalizes the levels of some mis-expressed genes in lungs of the rats exposed to hypoxia for four days**. A-C: levels of HIF target genes and PH related genes in the lungs of rats under normoxia or hypoxia, treated with control or HIF2 inhibitor PT2567 (N=6 for each group). **A**) Classical HIF target genes. **B**) Genes involved in inflammation. **C**) Genes involved in signaling and proliferation. Statistical significance determined by t-test.

Supplementary Figure 5. Hif2α, not Hif1α siRNA significantly prevents gene expression changes observed in normal pulmonary artery endothelial cells in response to acute hypoxia. To determine if HIF2 or HIF1 activity is responsible for hypoxia-mediated gene expression changes in EC, normal human pulmonary artery EC cells (N=3) were transfected with control or siRNAs targeting Hif1α or Hif2α mRNAs. Post-transfection of 16 hours, cells were exposed to normoxia or hypoxia (1.5% O2) for additional 16 hours, and then cells were collected for RNA preparation and qRT-PCR. **A**) Hif1α and Hif2α mRNA levels, to monitor the knockdown efficiency; **B**) Select classical HIF target genes; **C**) Genes involved in inflammation that are significantly induced by hypoxia in EC (Fig 6B); **D**) Genes involved in signaling and proliferation that are significantly altered by hypoxia in EC (Fig 6C). Statistical significance determined by t-test.
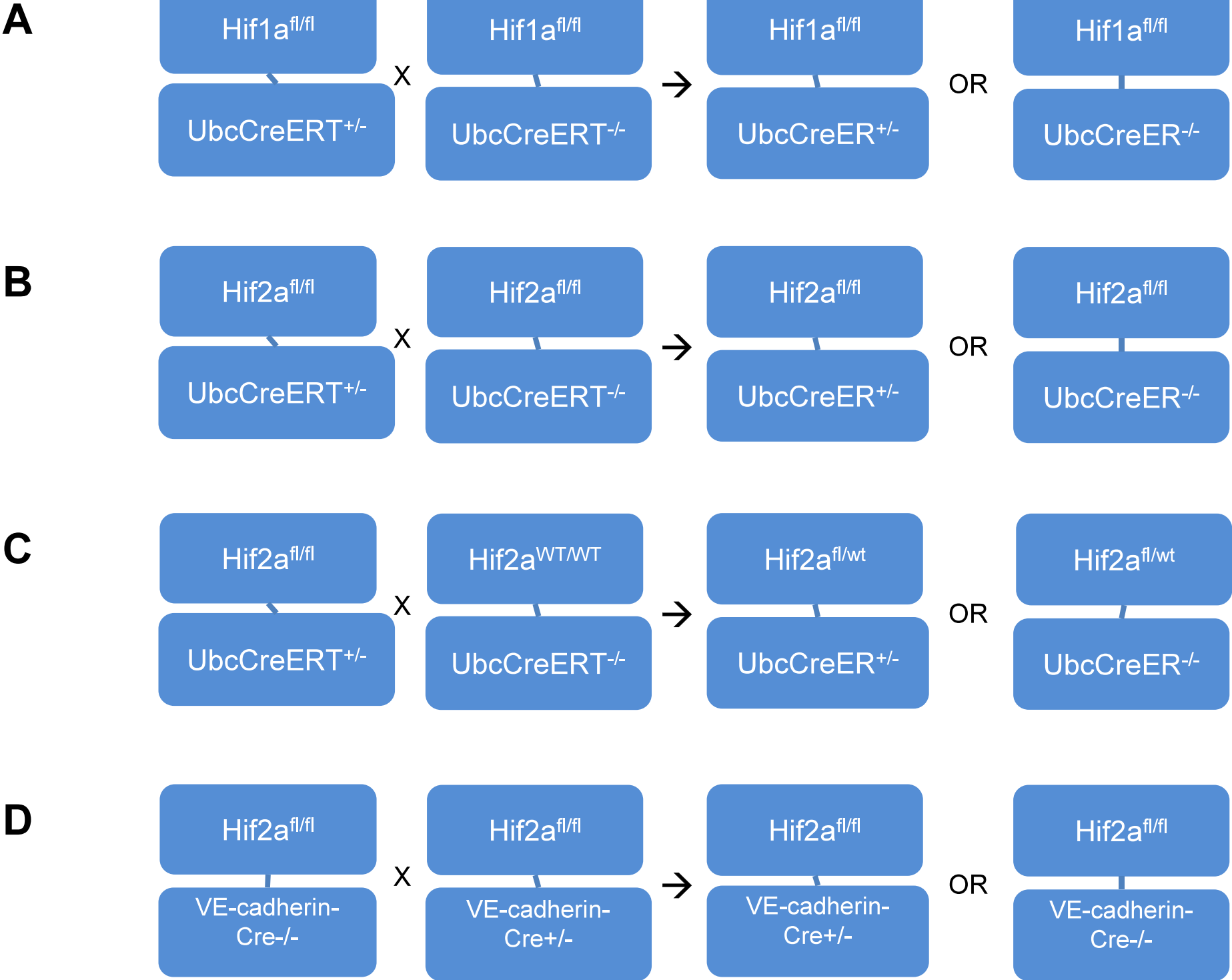
Supplementary Figure 6. Signals generated from normal pulmonary vascular EC under hypoxia, in a HIF2 activity dependent manner, significantly activate normal pulmonary vascular SMCs. Conditional medium prepared from normal pulmonary vascular ECs (N=3), cultured under normoxia or hypoxia (1.5% O2 for 24 hours), in the presence of DMSO or HIF2 inhibitor PT2567 (1 μM), were added to culture
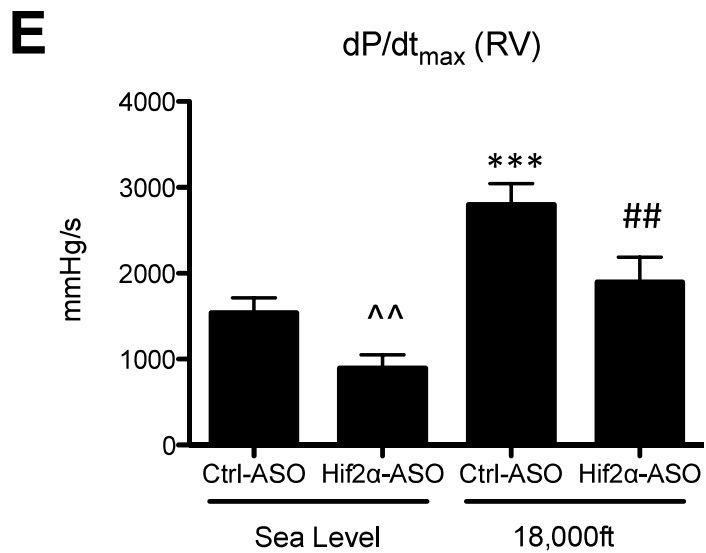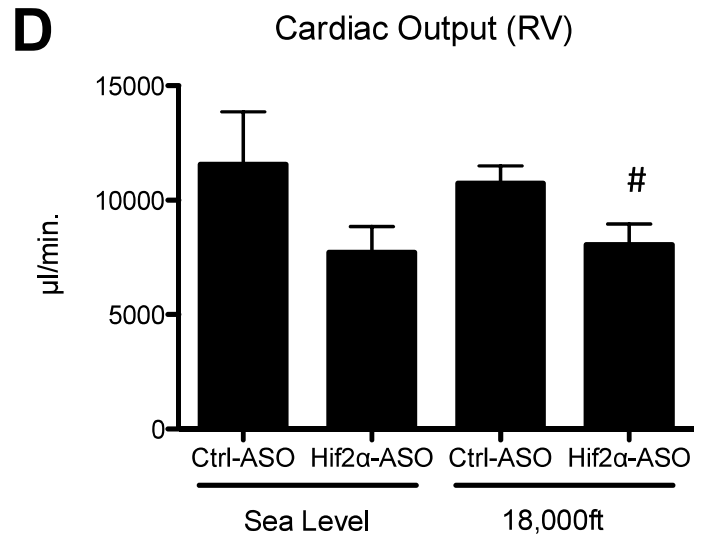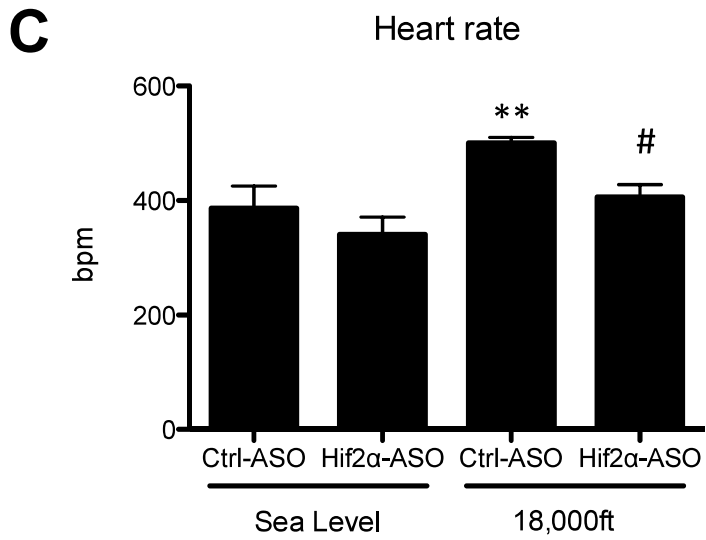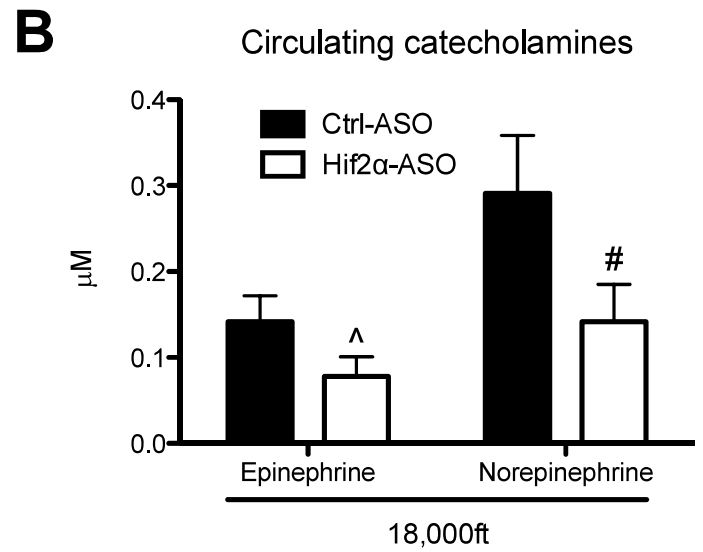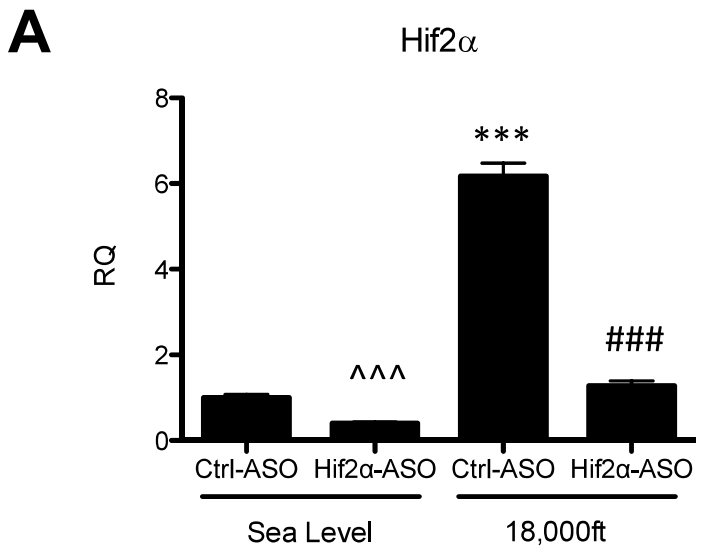
medium of normal pulmonary vascular SMCs (N=3) that were cultured under normaxia. After 24 hours, SMC cells were collected for RNAs that were used to examine the mRNA levels of the indicated genes involved in cell proliferation (CCNE1 and CCNE2), pro-inflammation (CCL2) and anti-apoptosis (BCL2, BCL2L1 and BIRC5). Statistical significance determined by t-test.

Supplementary Figure 7. **Endothelial deletion of Hif2α markedly attenuates some pathologic gene expression in the lungs of chronically (5 weeks) hypoxic mice. A-C**: levels of HIF target genes and PH related genes in the lungs of normoxic or hypoxic mice, with or without EC deletion of Hif2α gene. **A**) Classical HIF target genes. **B**) Genes involved in inflammation. **C**) Genes involved in signaling and proliferation. Statistical significance determined by t-test.

Supplementary Figure 8. **Endothelial deletion of Hif2α markedly attenuates pathologic gene expression in the RVs of chronically hypoxic mice.** In the hypertrophic heart, cardiac myocytes display an altered gene signature, such as isoform switching from α-myosin heavy chain (α-MHC, Myh6) to β-MHC (Myh7) and re-expression of skeletal muscle α-actin (Acta1). **A**) Increased expression of Acta1 was attenuated in hypoxic EC-Hif2α KO mice. **B**) Myh6 expression was reduced in hypoxia-exposed mice, but was not reversed in hypoxic EC-Hif2α KO mice. **C**) Increased expression of Myh7 was attenuated in hypoxic EC-Hif2α KO mice. Statistical significance determined by t-test.

Supplemental Figure 1

A

| Hif1a$^{fl/fl}$ | | Hif1a$^{fl/fl}$ | | Hif1a$^{fl/fl}$ | | Hif1a$^{fl/fl}$ |
| UbcCreERT$^{+/-}$ | X | UbcCreERT$^{-/-}$ | → | UbcCreER$^{+/-}$ | OR | UbcCreER$^{-/-}$ |

B

| Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ |
| UbcCreERT$^{+/-}$ | X | UbcCreERT$^{-/-}$ | → | UbcCreER$^{+/-}$ | OR | UbcCreER$^{-/-}$ |

C

| Hif2a$^{fl/fl}$ | | Hif2a$^{WT/WT}$ | | Hif2a$^{fl/wt}$ | | Hif2a$^{fl/wt}$ |
| UbcCreERT$^{+/-}$ | X | UbcCreERT$^{-/-}$ | → | UbcCreER$^{+/-}$ | OR | UbcCreER$^{-/-}$ |

D

| Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ | | Hif2a$^{fl/fl}$ |
| VE-cadherin-Cre-/- | X | VE-cadherin-Cre+/- | → | VE-cadherin-Cre+/- | OR | VE-cadherin-Cre-/- |

A Hif2α

B Circulating catecholamines

C Heart rate

D Cardiac Output (RV)

E dP/dt$_{max}$ (RV)

A Classical HIF target genes

B Inflammatory genes

PT rat 4 weeks

C Signaling

A Classical HIF target genes

Supplemental Figure 4

B Inflammatory genes
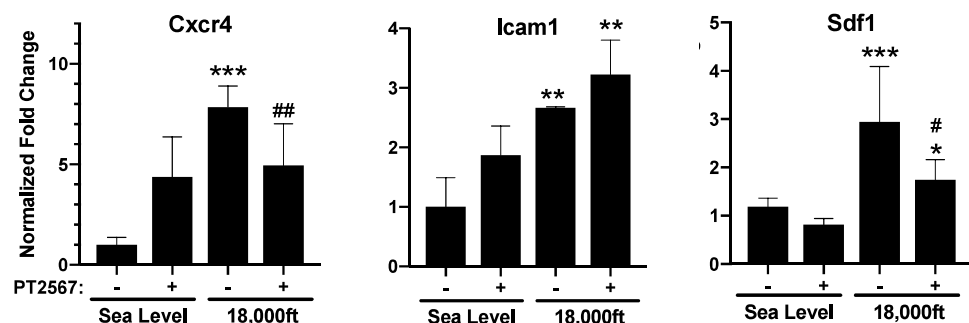
PT rat 4 days

C Signaling
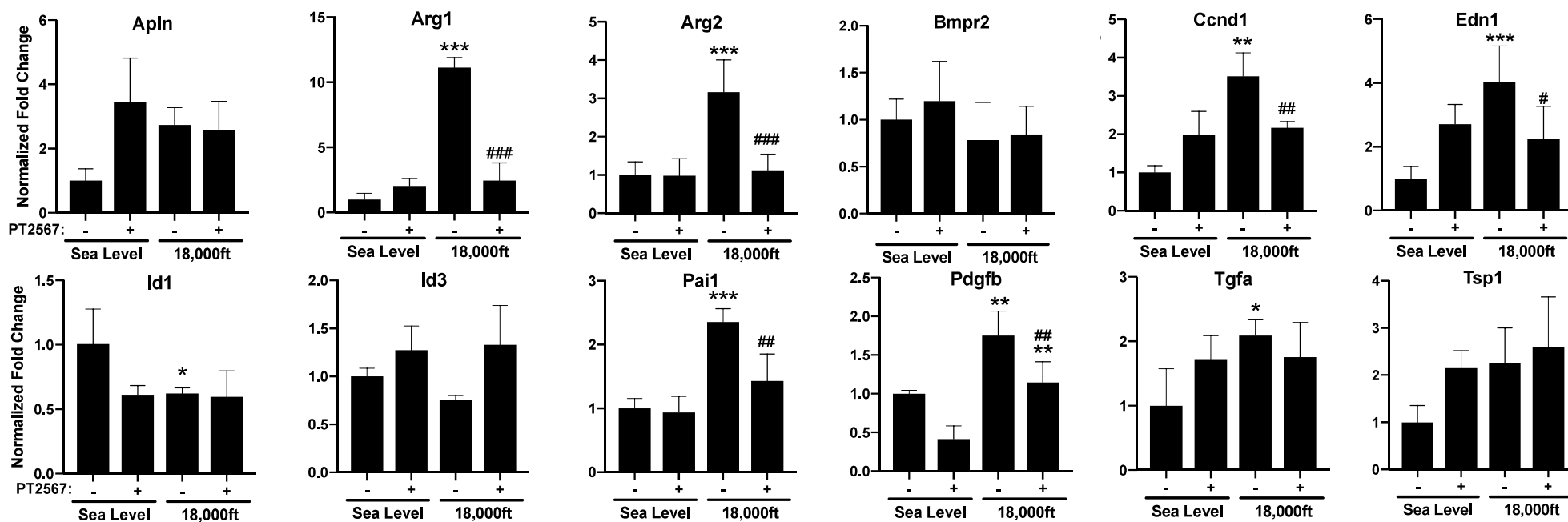
A HIF-a
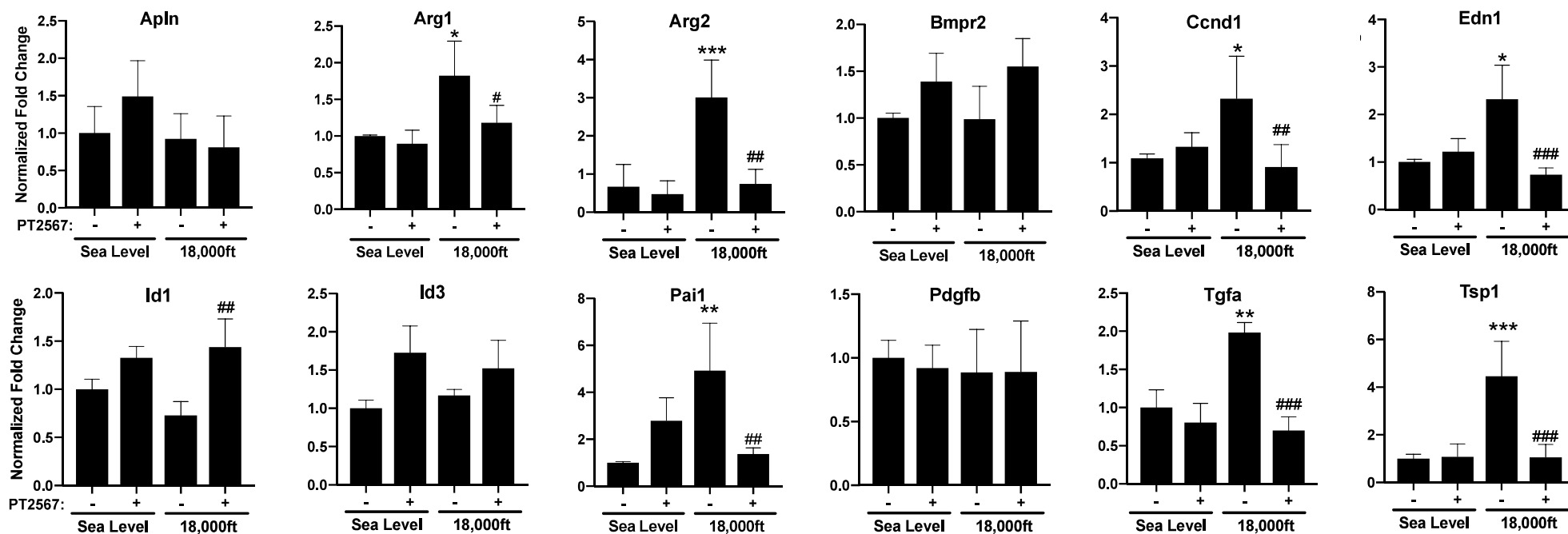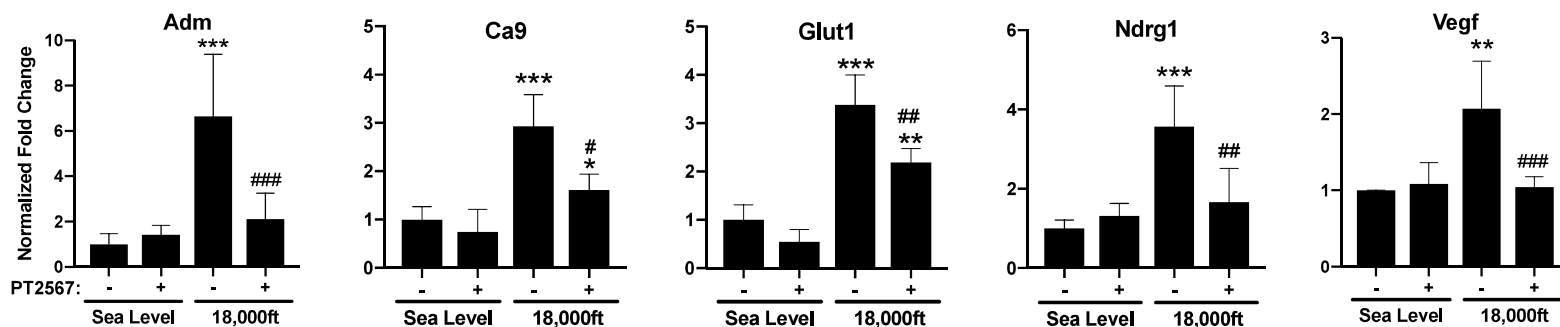
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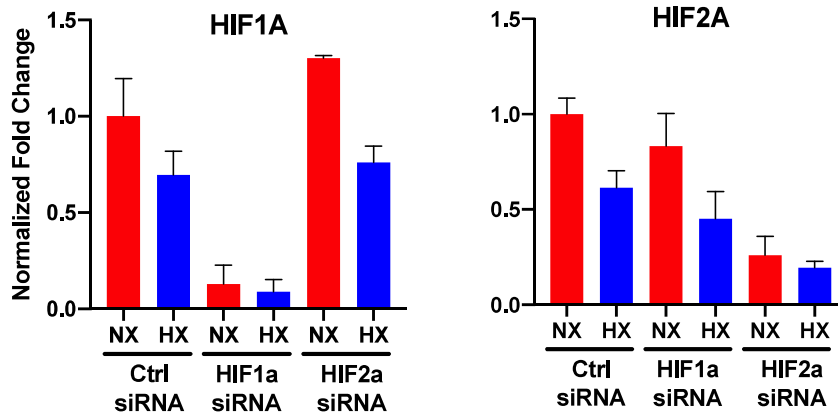
B Classical HIF target genes
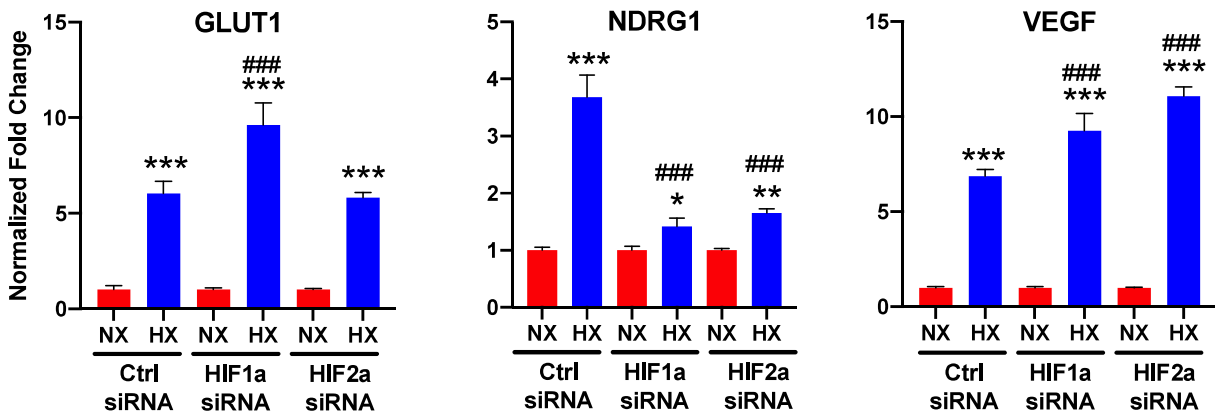
C Inflammatory genes
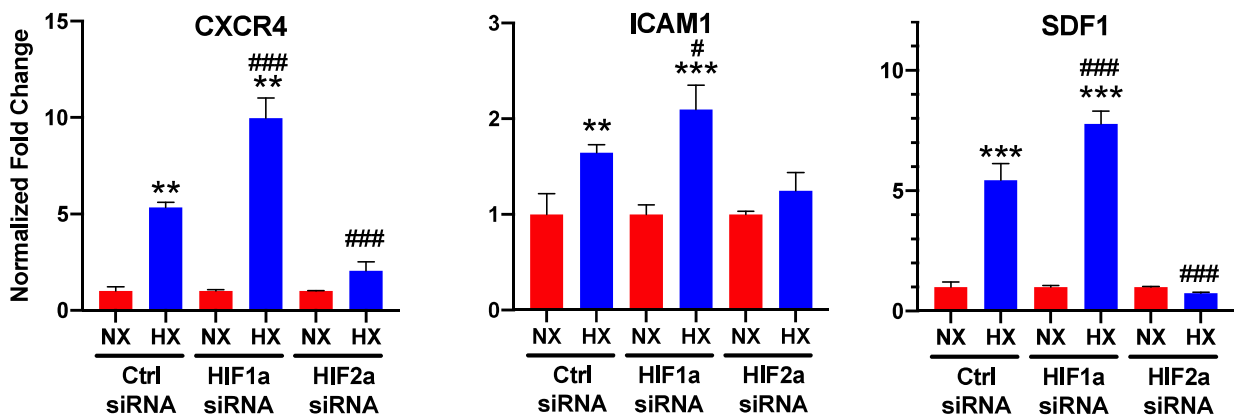
D Signaling

A **Classical HIF target genes**
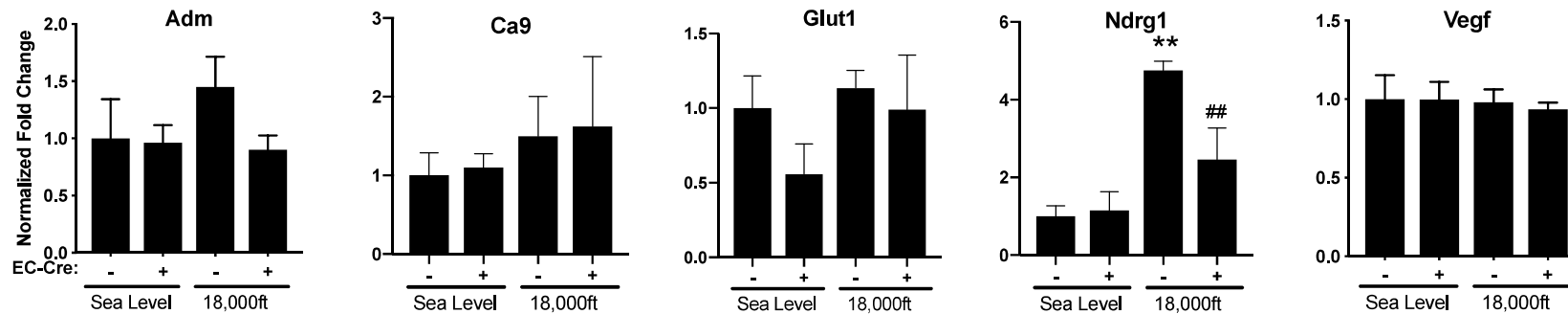
B **Inflammatory genes**

C **Signaling**

A Acta1

B Myh6

C Myh7
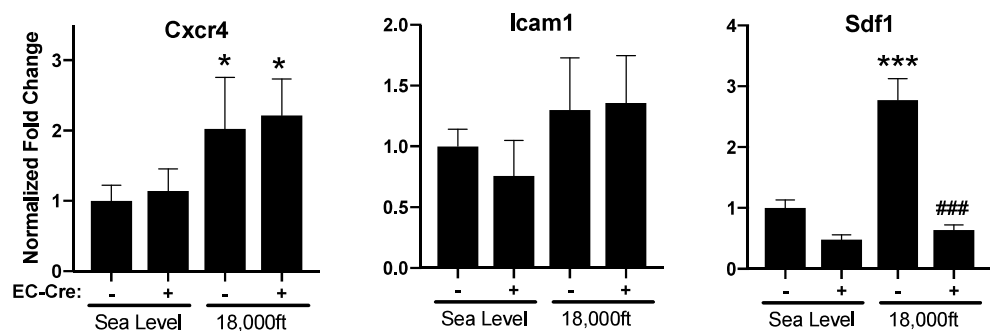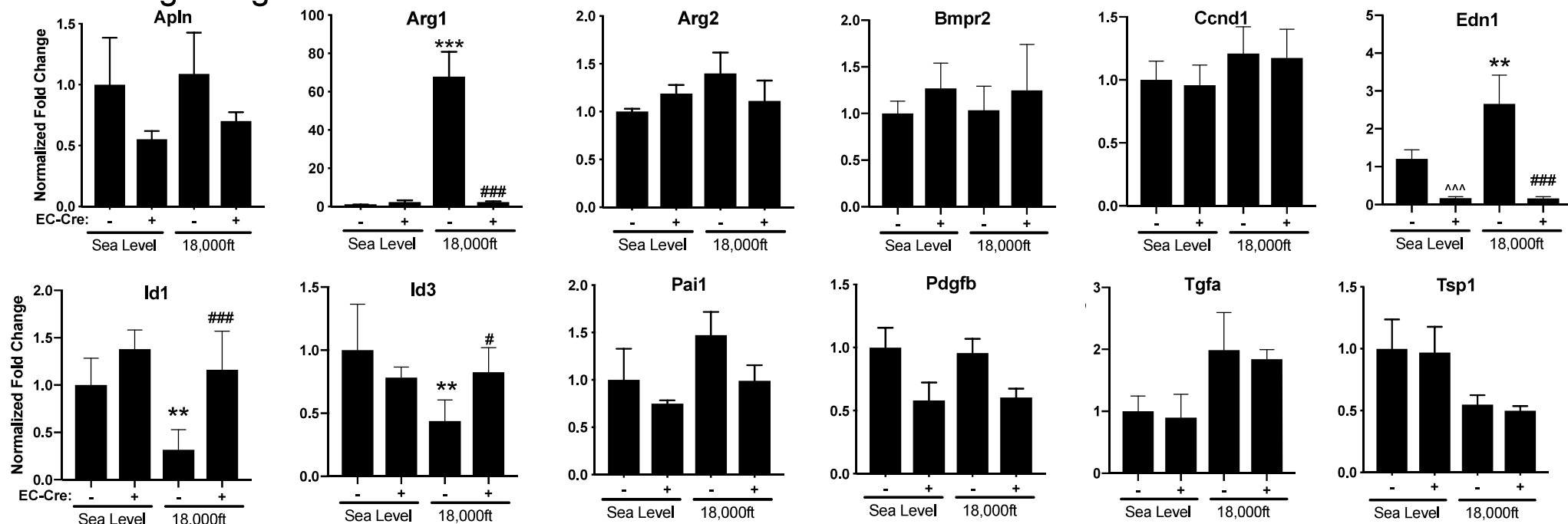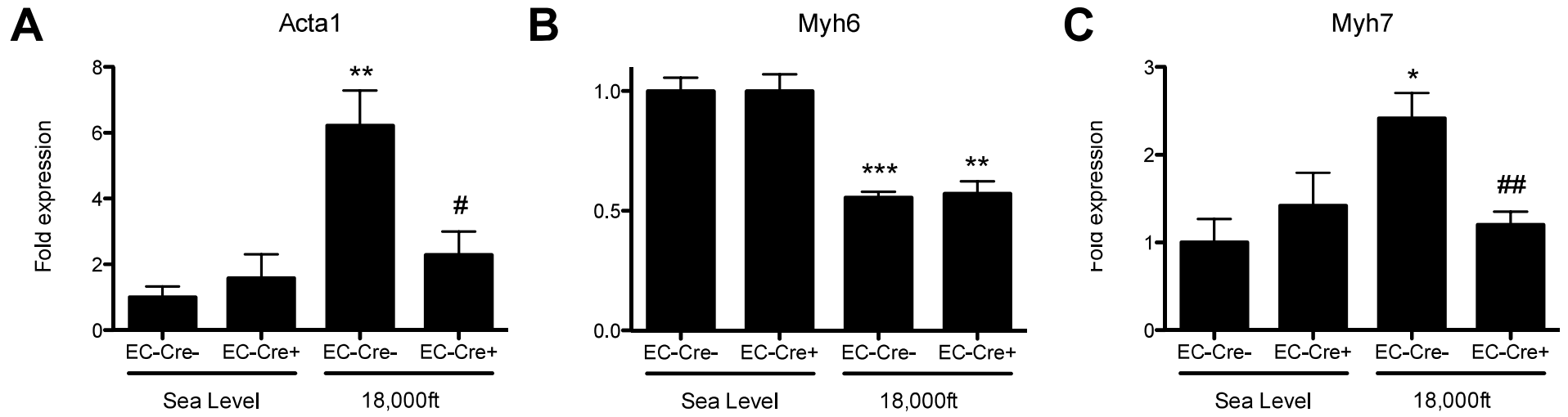
Mice	Reduction of mRNA
Hif1α$^{fl/fl}$;UbcCreERT$^+$	Hif1α: -80% ± 5%
Hif2α$^{fl/fl}$;UbcCreERT$^+$	Hif2α: -71% ± 6%
Hif2α$^{fl/WT}$;UbcCreERT$^+$	Hif2α: -36% ± 4%
Hif1α-ASO	Hif1α: -60% ± 5% (RV)
Hif2α-ASO	Hif2α: -72% ± 4%

Supplementary Table 1. Hif1a and HIF2a mRNA reduction in floxed and ASO treated mice. Rate of reduction in Hifa mRNA after hypoxia exposure (experimental setup: Fig. 1A and Fig. 3C)) in cre-positive mice and in ASO-treated animals, compared to the appropriate controls (cre-negative mice or Ctrl-ASO). Similar results were obtained for normoxic animals (data not shown). Expression was studied in lung tissue, with the exception of Hif1α ASO, for which RV tissue was used (no lung tissue available). There was no reduction of Hif1α, when Hif2α was targeted and vice versa.

	Sea Level		18,000ft	
	Hif1α$^{fl/fl}$; UbcCre-	Hif1α$^{fl/fl}$; UbcCre+	Hif1α$^{fl/fl}$; UbcCre-	Hif1α$^{fl/fl}$; UbcCre+
Weigth (g)	29.3	27.3	24.2 (**)	22.0 (**)(^)
Females / Males (n)	6/3	9/2	3/7	5/4
Heart Rate (bpm)	366	340	448 (***)	435 (*)

Supplementary Table 2. Parameters were taken after exposure to either sea level or high altitude at the end of the experiment. *, ** and ***: p<0.05, p<0.01 and p<0.001 for sea level vs. high altitude within the same genotype. ^p<0.1 for cre-negative vs. Cre-positive within the same condition. T-test was used.

	Sea Level		18,000ft	
	Hif2α$^{fl/WT}$; *UbcCre-*	Hif2α$^{fl/WT}$; *UbcCre+*	Hif2α$^{fl/WT}$; *UbcCre-*	Hif2α$^{fl/WT}$; *UbcCre+*
Weight (g)	25,0	26,3	23,5	23,1
Females / Males (n)	5/2	1/3	3/7	5/7
Heart Rate (bpm)	397,1	390,0	390,0	360,0

Supplementary Table 3. Parameters were taken after exposure to either sea level or high altitude at the end of the experiment.

	Sea Level		18,000ft	
	Ctrl-ASO	*Hif2a-ASO*	*Ctrl-ASO*	*Hif2a-ASO*
Weight (g)	27,6	26,2	23,3 (**)	22,4 (***)(^)
Females / Males (n)	0/8	0/8	0/8	0/7
Heart Rate (bpm)	386,7	340,5	500,5 (**)	406,4 (##)

Supplementary Table 4. Parameters were taken after exposure to either sea level or high altitude at the end of the experiment. **, ***: p<0.01 and p<0.001 for sea level vs. high altitude within the same ASO-treatment group. ^, ##: p<0.1 and p<0.01 for Ctrl-ASO vs. Hif2a-ASO within the same condition. T-test was used.

	Sea Level		18,000ft	
	Hif2α$^{fl/fl}$; EC-Cre-	Hif2α$^{fl/fl}$; EC-Cre+	Hif2α$^{fl/fl}$; EC-Cre-	Hif2α$^{fl/fl}$; EC-Cre+
Weight (g)	23,8	25,7	22,4	21,3
Females / Males (n)	5/4	4/4	4/3	6/1

Supplementary Table 5. Parameters were taken after exposure to either sea level or high altitude at the end of the experiment.

Gene		Mouse	Rat	Human
Acta1	fwd	AATGCTTCTAGGCGACTCG		
	rev	CCTGCAACCACAGCACGATT		
Adm	fwd	GACAAAGACAAGGACGGCATG	GGTATCGGAGCATCGCTACAG	GAG TGG AAG TGC TCC CCA CTT
	rev	GCCTTGAGGGCTGATCTTGTT	GAACGGCGAGCGAACCCAAT	CTT GTA CCA TGG GCG CCT A
Apln	fwd	GACCGAGTTGCAGCATGAATC	TGCCACTGATGCTGCCTCCA	ATGAATCTGCGGCTCTGCGT
	rev	TCAAGGAGAGCCAGAGCAGC	CTTCACCAGGTAGCGCATGTT	GCGGCATCAGGGACCCTCC
Arg1	fwd	GCTTGCGAGACGTAGACCCT	CAGAGGAGGTGACTCGTACTGTG	ACTCGAACAGTGAACACAGCAGT
	rev	TGCCAATCCCCAGCTTGTCT	ACAAGACAAGGTCAACGCCA	CAATAGGCTTGTGATTACCCTCC
Arg2	fwd	GAAGAGGCCAAGGCAACAGC	AGGCTGCACTCACTCGAGGT	AATCCTCAGTTGGCCACCTC
	rev	TGTCCTCCTTCTCTTGTCTGACC	ATGATTGGCAGGGAGGCGAT	CAATCACATCTACTGCCAGGTTAGC
Bcl2				TCCACCAAGAAAGCAGGAAAC
				TGGACAGGATAGCAGCACAGGA
Bcl2L1				CAG GAG AAC GGC GGC TGG GA
				TCG GCT GCT GCA TTG TTC CCA
Birc5				GTGGCTGCTTCTCTCTCTCT
				GCCTTCTTCCTCCCTCACTT

Supplementary Table 6. Primer sequences used for qRT-PCRs.

Gene		Mouse	Rat	Human
Bmpr2	fwd	AGAAGAGCACAGAGGCCCAAT	GTC TAC ACA GTG TGA GCA TGA TGG	TGCTATGCAGAATGAACGCAAC
	rev	CTGTATACTGCTGCCATCCAGG	CTC CAG CAG CTT CAG GTT ATC CAA	AAGGACCAATTTTTGGCACACG
Ca9	fwd	TGAAGGGTCTCTGACCACACC	CTGCCTCTCTGCTGGTGACA	GGT GTC ATC TGG ACT GTG TTT
	rev	ACACAGTCCAGATGACCCCC	AGGAGGAAGGCGATGCTGGT	GGT GTC AGA GAG GGT GTG GAG
Ccl2	fwd			CATGAAAGTCTCTGCCGCCCT
	rev			TGAGCGAGCCCTTGGGGAAT
Ccna1	fwd		CTTCAGTACTTAAGGCGGCAA	
	rev		GGCCAAATTCTCAGTCCTGATG	
Ccnd1	fwd	CGT GGC CTC TAA GATGAA GGA	ATGGTGGCTGCTGGGAGTGT	CGT GGC CTC TAA GATGAA GGA
	rev	GTA GAT GCA CAG CTT CTC GGC	TAGCAGGAGAGGAAGTTGTTGG	GTA GAT GCA CAG CTT CTC GGC
Ccne1	fwd			GGACACCATGAAGGAGGACG
	rev			TTCCTGGAGCGAGCCGAGAA
Ccne2	fwd			GTAGCTGGTCTGGCGAGGTT
	rev			ACGGCTACTTCGTCTTGACATTC
Cd68	fwd	CACAGGCAGCACAGTGGACA	CTTTGGATTCAAACAGGACCG	
	rev	CTTGGAGCTCTCGAAGAGATGA	GGTAGACTGTACTGTGGCTCTGATG	

Supplementary Table 6 (continued). Primer sequences used for RT-PCRs.

Gene		Mouse	Rat	Human
Cxcr4	fwd	CCCTCGCCTTCTTCCACTG	TGTTCCAGTTCCAGCACATC	ATCTGTGACCGCTTCTACCCC
	rev	CGAGGAAGGCATAGAGGATGG	CCTTGGAGTGTGACAGCTTG	AAACTGGAACACAACCACCCA
Edn1	fwd	GCACCGGAGCTGAGAATGGA	CGAGCTGAGAAGGAAGTGCAGAG	AAAGAGTGTGTCTACTTCTGCCACC
	rev	ACAGGAACAGCGCTTGGACC	AAGGAGGAGCAGGAGCAACG	CAACGTGCTCGGGAGTGTTG
Epo	fwd	TTGGAAAAGAATGGAGGTGGA		
	rev	GACAGGCCTTGCCAAACTTC		
Glut1	fwd	GGA GAG GTG TCA CCT ACA GC	GATGATGCGGGAGAAGAAGG	TGTGGGCCTTTTCGTTAACC
	rev	AAG GAT GCC AAC GAC GAT TC	AGCACCACGGCGATGAGGAT	ATCATCAGCATTGAATTCCGC
Hif1a	fwd	TGCAGCAAGATCTCGGCGAA		TTT TAC CAT GCC CCA GAT TCA
	rev	AGTGGCAACTGATGAGCAAGC		AGT GCT TCC ATC GGA AGG ACT
Hif2a (Epas1)	fwd	GACGGAGGTCTTCTATGAGTTGG		TACAAGGAGCCCCTGCTGTC
	rev	CGCATGATGGAGGCTTTGTC		TGCTGGATTGGTTCACACATG
Icam1	fwd	GAGGAGATACTGAGCCGCCA	CTTCCGACTAGGGTCCTGAA	CTGTATACTGCTGCCATCCAGG
	rev	GCTAGCCAGCACCGTGAATG	CTTCAGAGGCAGGAAACAGG	CTGGCTTCGTCAGAATCACGTT
Id1	fwd	GGTGGAGATCCTGCAGCATGTA	TTC TGC TCT ACG ACA TGA ACG GCT	GCACCCTCAACGGCGAGATCAGCG
	rev	CCGACTTCAGACTCCGAGTTCA	ACA TGC TGC AGT ATC TCC ACC TTG	CAGCGACACAAGATGCGATCGTCC

Supplementary Table 6 (continued). Primer sequences used for qRT-PCRs.

Gene		Mouse	Rat	Human
Id3	fwd	GGAACGTAGCCTGGCCATT	CTG TCG GAA CGT AGC CTA GCC ATT	CAG CGC GTC ATC GAC TAC ATT CTC
	rev	TCCAAGAGGCTAAGAGGCTCC	GCG AGT AGC AGT GGT TCA TGT CGT	CCA TCA GGG GGT CCA GGG GCT GGC
Myh6	fwd	GGGCAGCTCATGCGCATTGA		
	rev	TGCGCTCTTCAGCAGCGGTT		
Myh7	fwd	CAGCAGTTGGATGAGCGACT		
	rev	GATGCGTGCCTGAAGCTCCT		
Ndrg1	fwd	ATGCCTGGAACCCACACTGT	GTGGAGGTGGTGCACACGTA	GAA GCA AGC ATC TCC GCA TC
	rev	AACTACCAGCCAGAGCAGGGC	TTGTAGGCGCTGATGAACAGG	CAA CCG GCC ACT GGT TAA TG
Pai1	fwd	TTTGGACCCAGTTCCCACC	TTGTGAAGGAGGAACGCTGC	
	rev	TGAAAGTGTTGTGCCCTCCAC	ATTGGCTCTTGTTGGCTGTCG	
Pdgfb	fwd	AATGCTGAGCGACCACTCCA	CAGCAGAGCCTGCCGTAATC	GGCCGAGTTGGACCTGAACA
	rev	TACGGAGTCTCTGTGCAGCAGG	GAACACCTCTGTACGCGTCTTG	TCCAGCTCGCCTCCAGAGTG
Pgk1	fwd	GGAGAGTCCAGAGCGACCCT		
	rev	GCAACTTTAGCGCCTCCCA		
Sdf1	fwd	ATCGGTGGCTGCGAGCTGAA	TGTGCATTGACCCGAAATTA	AGA TTG TAG CCC GGC TGA AG
	rev	ACATGCCTGGGATGCTGCGT	TCCTCAGGGGTCTACTGGAA	TCG GGT CAA TGC ACA CTT GT

Supplementary Table 6 (continued). Primer sequences used for qRT-PCRs.

Gene		Mouse	Rat	Human
Tgfa	fwd	GGGAAGGAAATGTGTTCCCTG	CAACAAGTGCCCAGATTCCC	GAAGGGAAGAACCGCTTGCT
	rev	GGCTCAATGGGTCAGAGCAT	GGCTTCTCTTCCTGCACCAA	TGGGCTCTTCAGACCACTGTT
Tnc	fwd		TGCTGAAGGTTGTGGCTGTG	
	rev		ATTCAGGCTCAGAGCAGTTGG	
Tsp1	fwd	CAATTTTCAGGGGGTGCTGC	ACATCTGCTCTGTCACCTGTGG	CCTGTGATGATGACGATGA
	rev	CCGTTCACCACGTTGTTGTC	GGGTTGTTGCAGAGTCGGCT	CTGATCTGGGTTGTGGTTGTA
Vegf	fwd	TGACAAGCCAAGCGCGTGAG	GGGATCTTTCATCGGACCAGT	TGT GCC CAC TGA GGA GTC C
	rev	CTCCTCTTCCTTCATGTCAG	CGGTGTCTGTCTGTCTGTCCGT	GGT TTG ATC CGC ATA ATC TGC

House keeping:

Gene		Mouse	Rat	Human
18S	fwd	GAA TTC CCA GTA AGT GCG GG (for mouse, rat and human)		
	rev	GGG CAG GGA CTT AAT CAA CG (for mouse, rat and human)		
Actb	fwd	GCCCTGAGGCTCTTTTCCAG	CCAGCCATGTACGTAGCCATCCAG	GCCCTGAGGCACTCTTCCA
	rev	TGCCACAGGATTCCATACCC	TCACAATGCCAGTGGTACGACCAG	ATGCCACAGGACTCCATGC

Supplementary Table 6. **Forward and Reverse sequences of qPCR primers used in the manuscript, listed in mouse, rat, and human genes**. The amplicon sizes are typically 50-80 mer and without significant secondary structures. All primer sets were validated by melt curve analysis, cloning and sequencing of qPCR products, and standard dilution assay to ensure specific PCR products and to have amplification efficiencies between 90% and 110%. *Hif1α* floxed mice and *Hif-2α* floxed mice were constructed wherein exon 2 (coding DNA binding domain) of the Hif1α or Hif2α gene was floxed with LoxP sites. To assess HIF1a and HIF2a deletion in HIF flox mice, qPCR primers for mouse HIF1a and HIF2a mRNAs are located in the exon 2 of HIF1α or HIF2α gene.

	Gene	Cell type	Normoxia	Hypoxia
Hypoxic induction of classical HIF target genes In normal pulmonary artery vascular cells	ADM	EC	100%±9.7	250.7%±24.3***
		Fib	100%±0.6	221.3%±41.2***
		SMC	100%±22.3	93.7%±6.8
	CA9	EC	100%±10.1	1328.6%±274.4*
		Fib	100%±18.6	19164.2%±3575.7**
		SMC	100%±26.7	235.1%±26.7
	GLUT1	EC	100%±27	301.3%±33***
		Fib	100%±1.6	477.0%±8.7***
		SMC	100%±20.2	461.7±140.2***
	NDRG1	EC	100%±8.6	301.8%±15.6***
		Fib	100%±8.7	333.5%±30.3***
		SMC	100%±9.6	312.6%±13.6***
	VEGF	EC	100%±8.2	193.5%±6.9***
		Fib	100%±5.3	208.5%±27.1***
		SMC	100%±7.2	209.6%±30***
Hypoxic induction Of inflammatory genes in normal pulmonary artery vascular cells	CXCR4	EC	100%4.8	250.0%±13.2***
		Fib	100%±2.9	46.3%±23.9
		SMC	100%±3.3	195.6%±18.2***
	SDF1	EC	100%±13.9	321.5%±13.9***
		Fib	100%±0.61	123.2%±53.0
		SMC	100%±3.3	109.4%±32.42
	ICAM1	EC	100%±23.6	307.4%±18.8***
		Fib	100%±3.7	138.6%±4.4
		SMC	100%±41.6	162.7%±18.8

Supplementary Table 7. Gene expression in pulmonary artery cells by qRT-PCR, as in Fig. 6. Statistical significance as determined by t-test.

Gene	Cell type	Normoxia	Hypoxia
	EC	100%±12.0	208.3%±57.7***
APLN	Fib	100%±52.7	595.8%±64.5***
	SMC	100%±4.3	530.%±16.4***
	EC	100%±30.8	89.4%±15.4
ARG1	Fib	100%±21.3	89.8%±32.3
	SMC	100%±39.8	82.7%±4.9
	EC	100%±22.4	97.9%±11.2
ARG2	Fib	100%±15.5	79.0%±10.1
	SMC	100%±8.7	87.2%±27.3
	EC	100%±16.5	94.7%±17.1
BMPR2	Fib	100%±25.1	127.8%±15.4
	SMC	100%±27.0	76.6%±15.0
	EC	100%±5.1	103%±6.1
CCND1	Fib	100%±9.6	90%±5.8
	SMC	100%±30.8	110%±32.7
	EC	100%±13.1	118.0%±22.7
EDN1	Fib	100%±38.3	366.5%±17.4***
	SMC	100%±25.0	144.7%±8.9***
	EC	100%±14.7	30.0%±4.3***
ID1	Fib	100%±12.3	83.6%±12.3
	SMC	100%±14.4	96%±7.1
	EC	100%±4.6	54.8%±12.0***
ID3	Fib	100%±4.3	96.4%±6.0
	SMC	100%±10.0	91.2%±1.2

The leftmost column spanning all rows reads: "Hypoxic induction Of signaling factors in normal pulmonary artery vascular cells"

Supplementary Table 7, continued.

	Gene	Cell type	Normoxia	Hypoxia
Hypoxic induction Of signaling factors in normal pulmonary artery vascular cells (continued)	PDGFB	EC	100%±25.7	161.2%±23.3*
		Fib	100%±33.5	120.1%±12.0
		SMC	100%±43.2	257.3%±39.6**
	TGFA	EC	100%±17.8	775.3%±127.1***
		Fib	100%±23.0	80%±15.0
		SMC	100%±60.0	346.9%±16.9***
	TSP1	EC	100%±20.1	104.0%±4.6
		Fib	100%±8.1	141.7%±28.1
		SMC	100%±30.0	159.9%±62.5*

Supplementary Table 7, continued.

	Gene		Normoxia	Hypoxia	Δ
Hypoxic induction of classical HIF target genes in EC	GLUT1	DMSO	100.0%±1.2	721.26%±75.1***	
		PT2567 0.1µM	103.0%±13.3	555.5%±20.8***	23% #
		PT2567 1µM	88.9%±14.62	346.8%±21.2**	36.5% ###
		PT2567 10µM	116.7%±19.6	242.6%±84.4*	51.9% ###
	NDRG1	DMSO	100.0%±12.6	603.3%±39.7***	
		PT2567 0.1µM	94.5%±21.7	578.3%±23.9***	4,10%
		PT2567 1µM	96.2%±13.2	400.0%±57.8***	33.7% ##
		PT2567 10µM	99.4%±8.1	522.4%±7.0***	13.4% #
	VEGF	DMSO	100.0%±30.9	516.0%±87.8***	
		PT2567 0.1µM	97.1%±20.6	384.4%±50.4***	25.5% #
		PT2567 1µM	114.8%±18.0	271.0%±54.0**	47.5% ###
		PT2567 10µM	92.6%±20.9	276.0%±33.6**	46.5% ###
Hypoxic induction of inflammatory genes in EC	CXCR4	DMSO	100.0%±15.0	350.0%±31.9**	
		PT2567 0.1µM	127.9%±26.3	319.3%±73.3**	8,77%
		PT2567 1µM	79.9%±1.01	85.9%±11.05	75.5% ##
		PT2567 10µM	110.5%±18.0	98.2%±26.7	71.9% ##
	ICAM1	DMSO	100.0%±33.1	450.8%±41.8**	
		PT2567 0.1µM	109.5%±60.0	105.6%±51.1	76.6% ##
		PT2567 1µM	116.9%±35.4	115.9%±10.2	74.3% ##
		PT2567 10µM	122.8%±15.5	143.2%±19.2	68.2% ##
	SDF1	DMSO	100.0%±23.6	395.8%±18.3**	
		PT2567 0.1µM	184.6%±10.3	424.6%±1.02**	107,30%
		PT2567 1µM	59.0%±31.3	45.5%±133.3	88.5% ###
		PT2567 10µM	99.1%±28.4	115.5%±4.74	70.8% ###

Supplementary Table 8. Gene expression in EC by qRT-PCR, as in Fig. 7. Δ (inhibitory rate) refers how effective of HIF2 inhibitor PT2567 in reducing the induction of the gene by the hypoxia. Statistical significance as determined by t-test.

	Gene		Normoxia	Hypoxia	Δ
Hypoxic induction of signaling factors in EC	ID1	DMSO	100.0%±25.0	30.1%±4.3**	
		PT2567 0.1μM	107.5%±24.3	48.5%±10.1*	161,00%
		PT2567 1μM	134.5%±35.4	63.0%±3.3***	209.3% ##
		PT2567 10μM	82.9%±0.53	69.4%±8.2	230.6% ##
	ID3	DMSO	100.0%±4.6	57.8%±10.7**	
		PT2567 0.1μM	77.7%±31.2	63.7%±20.2	110,20%
		PT2567 1μM	89.4%±25.5	74.2%±12.6	128,40%
		PT2567 10μM	71.9%±10.05	77.3%±5.95	124,40%
	TGFA	DMSO	100.0%±46.4	384.7%±64.9***	
		PT2567 0.1μM	139.2%±22.04	180.7%±5.8***	53.02% ###
		PT2567 1μM	147.6%±78.6	134.7%±3.1	65% ###
		PT2567 10μM	185.5%±158.8	140.4%±10.5	63.5% ###

Supplementary Table 8, continued.