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Suppression of HIF2 signaling attenuates the initiation of hypoxia-induced pulmonary hypertension

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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 HIF1 α deletion in mice did not prevent hypoxia-induced PH at 5 weeks. Mice with global HIF2 α deletion did not survive long-term hypoxia. Partial HIF2 α gene deletion, or HIF2-ASO

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(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 muscularization, 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 HIF2 α 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.

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.

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 Hif1 α or Hif2 α 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

Hif1 α is dispensable in establishing hypoxia-induced PH at 5 weeks in adult mice

To delete the Hif1 α gene in adult mice globally, Hif1 α ^{fl/fl};UbcCreERT⁺ and Hif1 α ^{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 Hif1 α 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 Hif1 α ^{fl/fl};UbcCreERT⁻ and Hif1 α ^{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 Hif1 α 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 Hif2 α do not survive long-term hypoxia

To investigate the role of *Hif2 α* in hypoxia-induced PH development in normal adult mice, adult Hif2 α ^{fl/fl};UbcCreERT⁺ and Hif2 α ^{fl/fl};UbcCreERT⁻ mice were similarly treated with tamoxifen and exposed to normoxia or hypoxia, as we did for *Hif1 α* mice. The efficiency of Hif2 α gene deletion in hypoxic Hif2 α ^{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 Hif2 α ^{fl/fl};UbcCreERT⁻ mice, Hif1 α ^{fl/fl};UbcCreERT⁺ mice and Hif1 α ^{fl/fl};UbcCreERT⁻ animals (Fig 1 and Fig 2A), all Hif2 α ^{fl/fl};UbcCreERT⁺ mice died within 4 weeks of hypoxic exposure (Fig 2A). This data indicates that global Hif2 α , but not Hif1 α , deletion is not compatible with survival of mice exposed to chronic hypoxic conditions.

Global partial Hif2 α deletion diminishes development of hypoxia-induced PH at 5 weeks in adult mice

To prevent lethality, we generated Hif2 α ^{wt/fl};UbcCreERT⁺ and Hif2 α ^{wt/fl};UbcCreERT⁻ mice, then treated them with tamoxifen and exposed them to hypoxia for 5 weeks. The efficiency of Hif2 α gene deletion in hypoxic Hif2 α ^{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 Hif2 α ^{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 Hif2 α attenuates development of PH induced by 5 weeks of hypoxia exposure.

Knockdown of Hif2 α utilizing antisense-oligonucleotides significantly reduces development of hypoxia-induced PH at 5 weeks in adult mice

To further investigate the role of Hif2 α in hypoxia-induced PH in normal adult mice and to examine whether targeting Hif2 α 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 Hif2 α ASO in reducing Hif2 α expression in adult mice maintained under normoxia, was tested in a pilot experiment, which showed an up to 90% reduction of Hif2 α mRNA levels in lungs, spleens, kidneys and livers in mice treated with Hif2 α -ASO two times per week for two weeks (Fig 3A left), without significant changes of Hif1 α mRNA in all organs examined (Fig 3A right). Further, Hif2 α -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 Hif2 α -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 Hif2 α gene deletion in hypoxic mice targeted with Hif2 α ASO was 72% at the end of the experiment (Suppl. Table 1). We did not observe any lethality in Hif2 α -ASO treated mice under hypoxia. However, hypoxic Hif2 α -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 HIF2 α 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 Hif2 α -ASO treated mice was lower, both, under normoxia and under hypoxia (Fig 3D). Under hypoxic conditions, Hif2 α -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 Hif2 α -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 Hif1 α -ASO reduced Hif1 α 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 Hif1 α 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 HIF2 α 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 *Ndr1* 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 inhibition 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 Hif2 α 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*, *Ndr1*, 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).

Hif2 α expression in endothelial cells is required for hypoxia-induced PH and vascular remodeling in mice

To confirm the essential role of EC Hif2 α in PH development in vivo, and to better understand the role of EC HIF2 in PH development, we generated and exposed EC Hif2 α knockouts and their controls to normoxia or hypoxia for 5 weeks. As expected, Hif2 α ^{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 Hif2 α ^{fl/fl}-EC-Cre⁺ mice, confirming previous reports that endothelial Hif2 α is not required for hypoxia-induced erythropoiesis and erythrocytosis (4). However, hypoxia exposed Hif2 α ^{fl/fl}-EC-Cre⁺ mice exhibited no signs of PH (Fig 8B–D). The increase in the number of α SMA-positive pulmonary vessels in hypoxia-exposure HIF2 α ^{fl/fl}-EC-Cre⁻ mice was also abolished in hypoxia-exposure Hif2 α ^{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 Ndr1, 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 Hif2 α 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 Hif2 α ^{fl/fl}-EC-Cre⁺ mice (Suppl Fig 8), compared to RV of hypoxic Hif2 α ^{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 HIF2 α , but not HIF1 α , 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 Hif2 α -deletion or activation in developing embryos. Although we also used mice with constitutive EC-specific knockout of Hif2 α , initiated in the embryo, we recognize that results from the EC HIF2 α KO study have limitations. However, the purpose of our EC HIF2 α 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 Hif1 α 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 HIF1 α expression. Additional *in vivo* studies in which HIF1 is activated by genetic manipulation of PHDs or the VHL protein, demonstrated Hif1 α 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 embryos 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 indispensable role of Hif1 α for PH in animals after 4–5 weeks of hypoxia exposure. However, we cannot exclude a transient role of Hif1 α in the earlier stage of PH development, which we did not investigate. Indeed, a transient role of Hif1 α in hypoxia-induced PH has been reported (1). Also, we cannot exclude disease-relevant Hif1 α -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 Hif1 α 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 Hif2 α , especially if Hif2 α also plays important roles in other cell types for other processes. Indeed, complete global deletion of Hif2 α 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 Hif2 α KO mice and mice/rats treated with Hif2 α inhibitor are beyond the scope of this study, we speculate that mice with significant loss of Hif2 α might have succumbed to cardiogenic shock under hypoxic conditions. This is based on our findings that Hif2 α -ASO-treatment reduced levels of circulating catecholamines that were significantly increased in hypoxia in control-ASO mice (Suppl. Fig. 2B). Hif2 α -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). Hif2 α 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 Hif2 α 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 Hif2 α -ASO-treated animals in addition to the observed changes in cardiac function, could result in critically low delivery of oxygen (DO₂) and finally death of Hif2 α KO mice. This interpretation of our data is consistent with previous studies, demonstrating Hif2 α -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 Hif2 α knockout, demonstrating that the observed reduction in RVSPs in Hif2 α -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 Hif2 α 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 (I_d 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 Hif2 α in SMC does not prevent hypoxic PH (7), studies on the effect of Hif2 α 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 maintenance 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 targets because there are many additional changes occur during cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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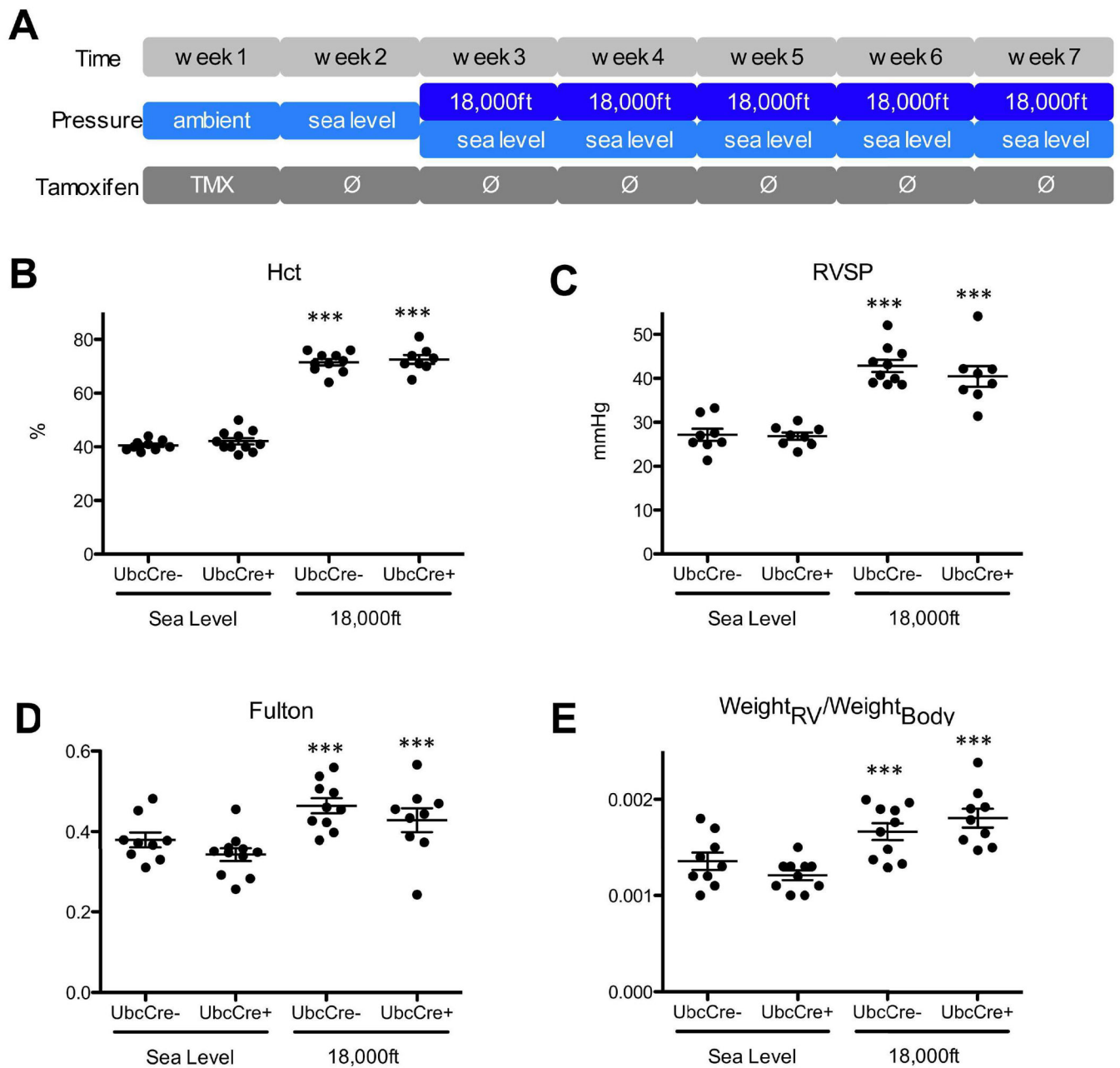


Figure 1: Global Hif1 α is dispensable in establishing hypoxia-induced PH at 5 weeks in adult mice.

A) Experimental setup: Tamoxifen was injected into mice daily during week 1 to activate Cre and delete Hif1 α 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). **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.

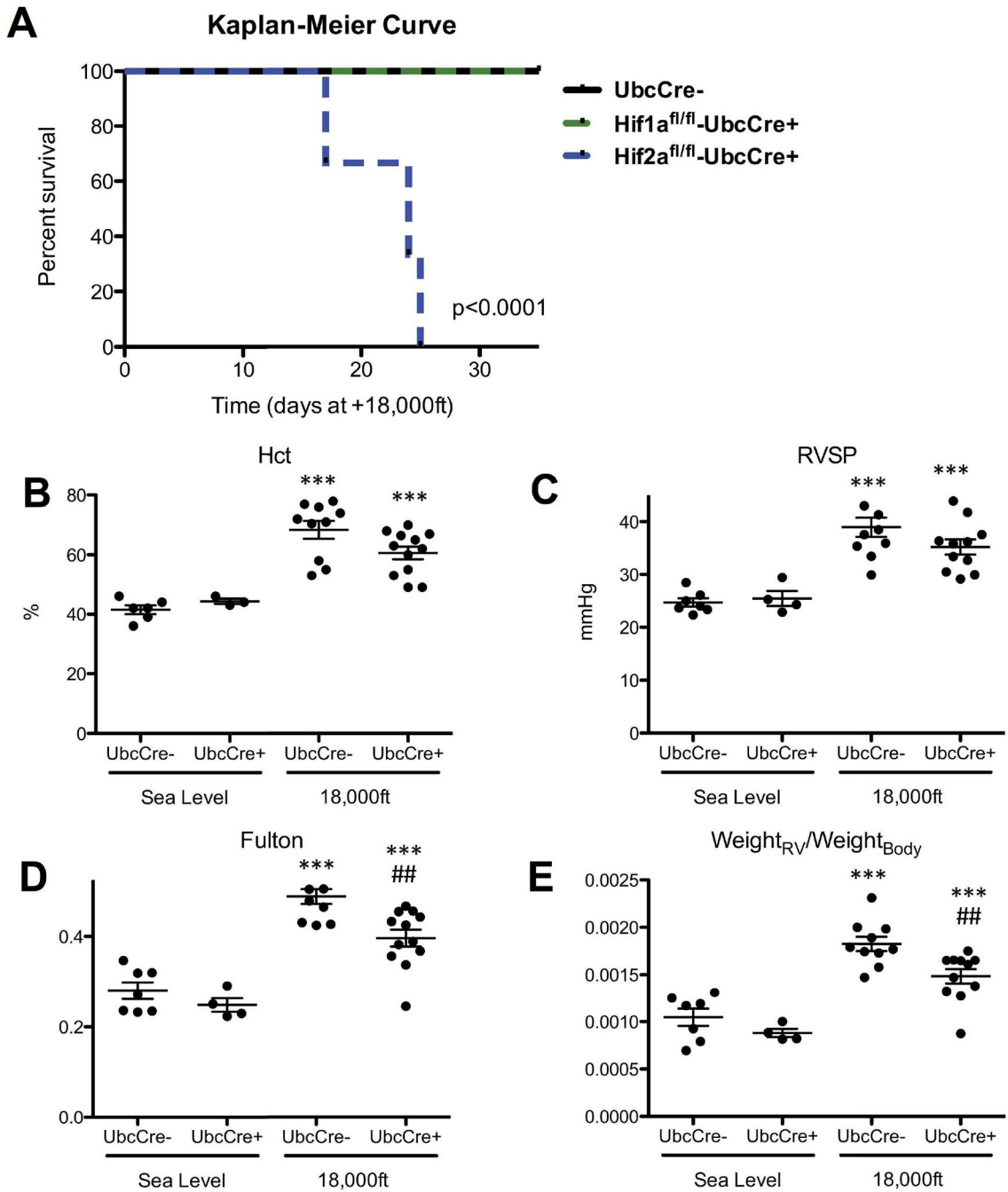
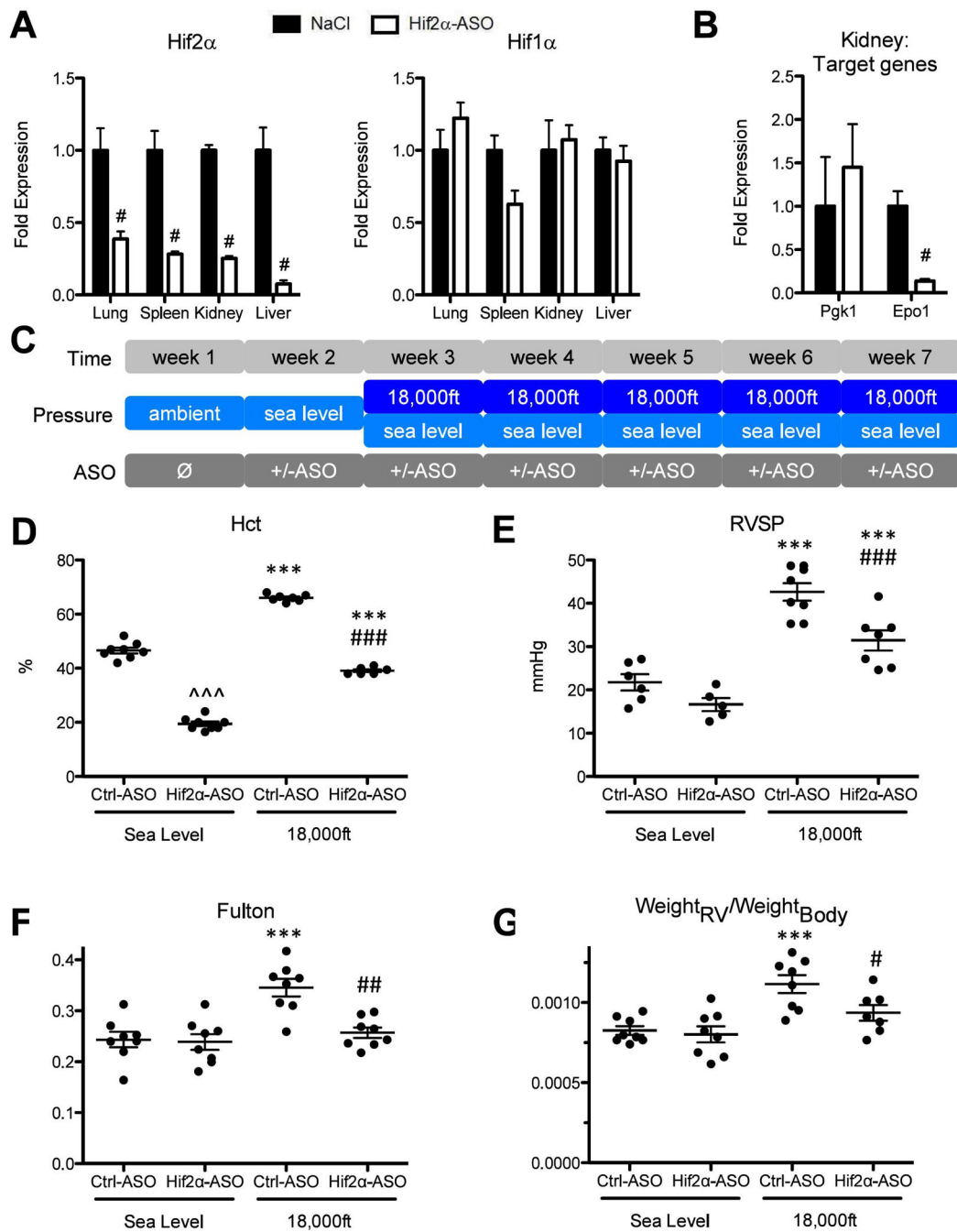


Figure 2: Global Hif2 α gene deletion is incompatible for mouse survival under hypoxic conditions while global partial Hif2 α gene deletion diminishes hypoxia-induced PH development at 5 weeks in adult mice.

Mice were treated with tamoxifen and exposed to normoxia or hypoxia as described in Fig 1A. **A**) Kaplan-Meier-Curve for survival of *Hif2 α ^{fl/fl};UbcCreERT⁺* mice during hypoxia exposure, compared to survival of *Hif1 α ^{fl/fl};UbcCreERT⁺* and *Hif2 α ^{fl/fl};UbcCreERT⁻* mice during exposure to hypoxia. More than 9 mice in each group were used for this experiment. **B–E**: Hemodynamics of *Hif2 α ^{fl/WT};UbcCreERT⁺* and *Hif2 α ^{fl/WT};UbcCreERT⁻* 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 $p < 0.05$).



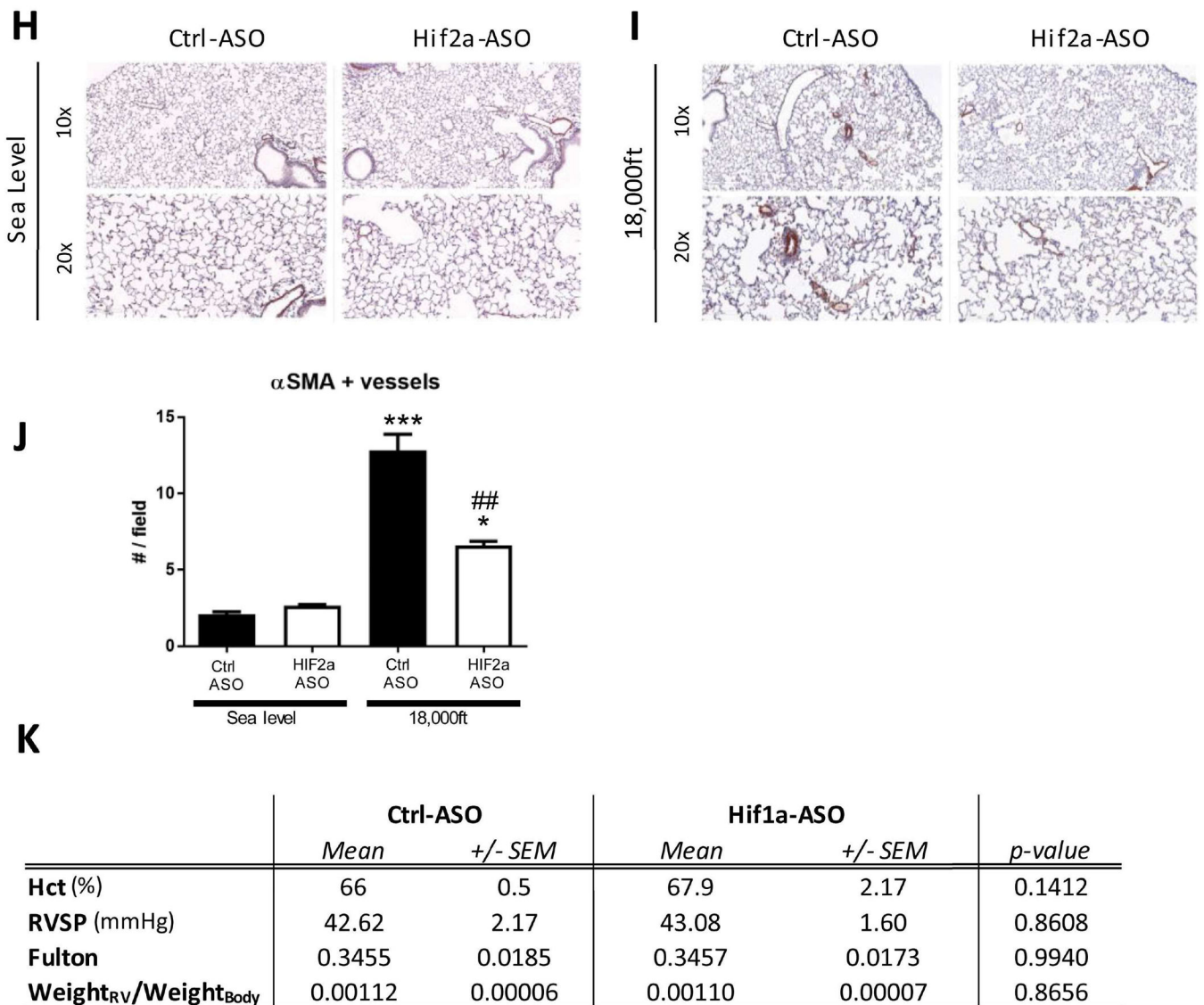
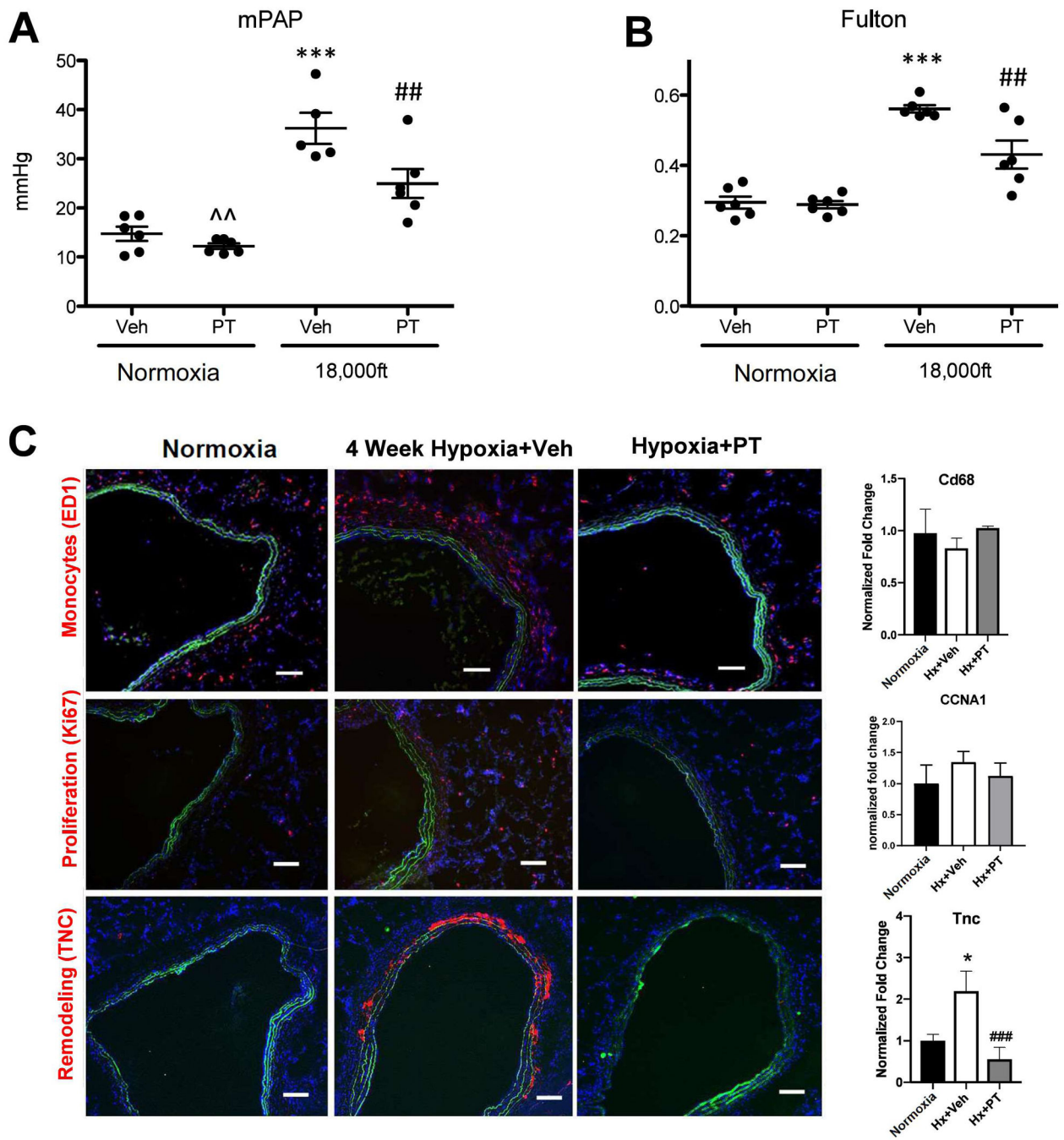


Figure 3: Knockdown of Hif2 α but not Hif1 α utilizing antisense-oligonucleotides significantly reduces development of hypoxia-induced PH at 5 weeks in adult mice.

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 Hif2 α mRNA (Hif2 α -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 Hif1 α and of Hif2 α 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 Hif2 α -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, Hif1 α -ASO, or Hif2 α -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 Hif1 α -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.



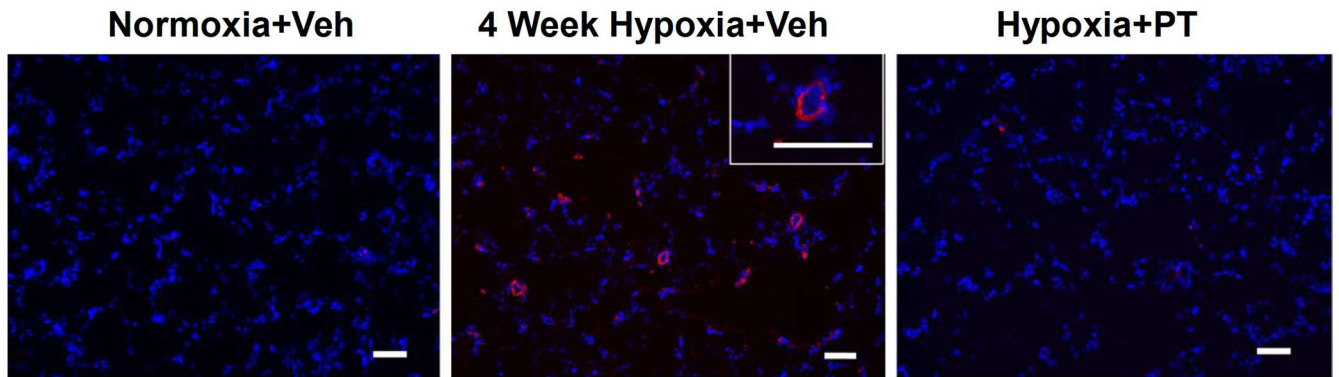
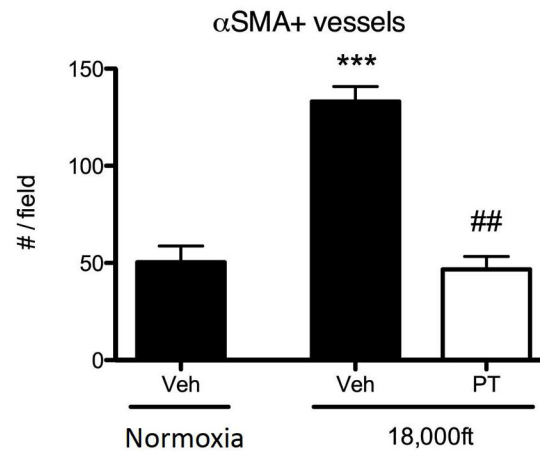
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Figure 4: Small molecule Hif2 inhibitor PT2567 significantly attenuates development of hypoxia-induced PH at 4 weeks in adult rats.

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: 100 μ m. Statistical significance determined by 2-way ANOVA (A, B) or by t-test (C–E).

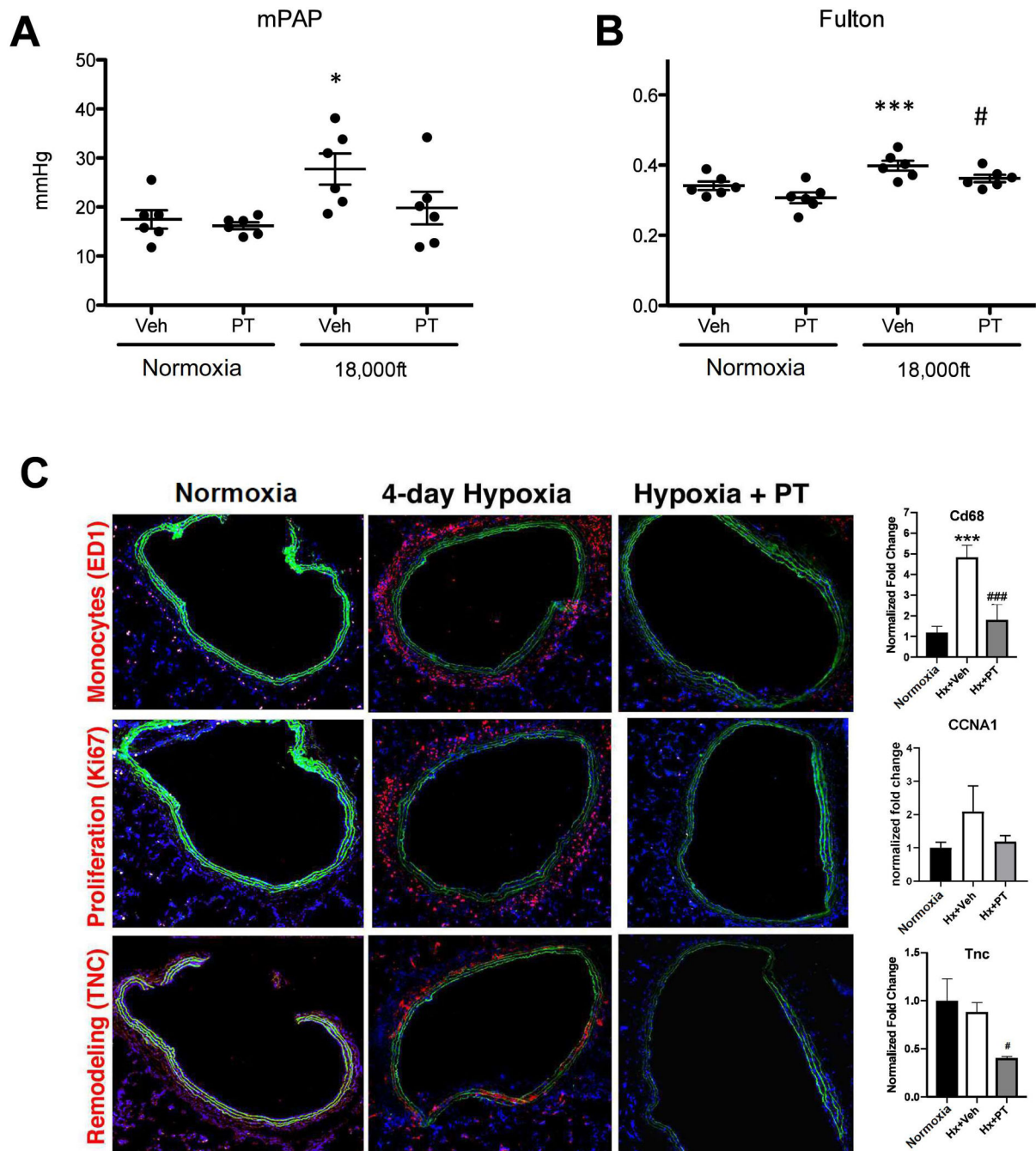


Figure 5: Small molecule Hif2 inhibitor PT2567 significantly attenuates early events in hypoxia-exposed adult rats at 4 days.

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).

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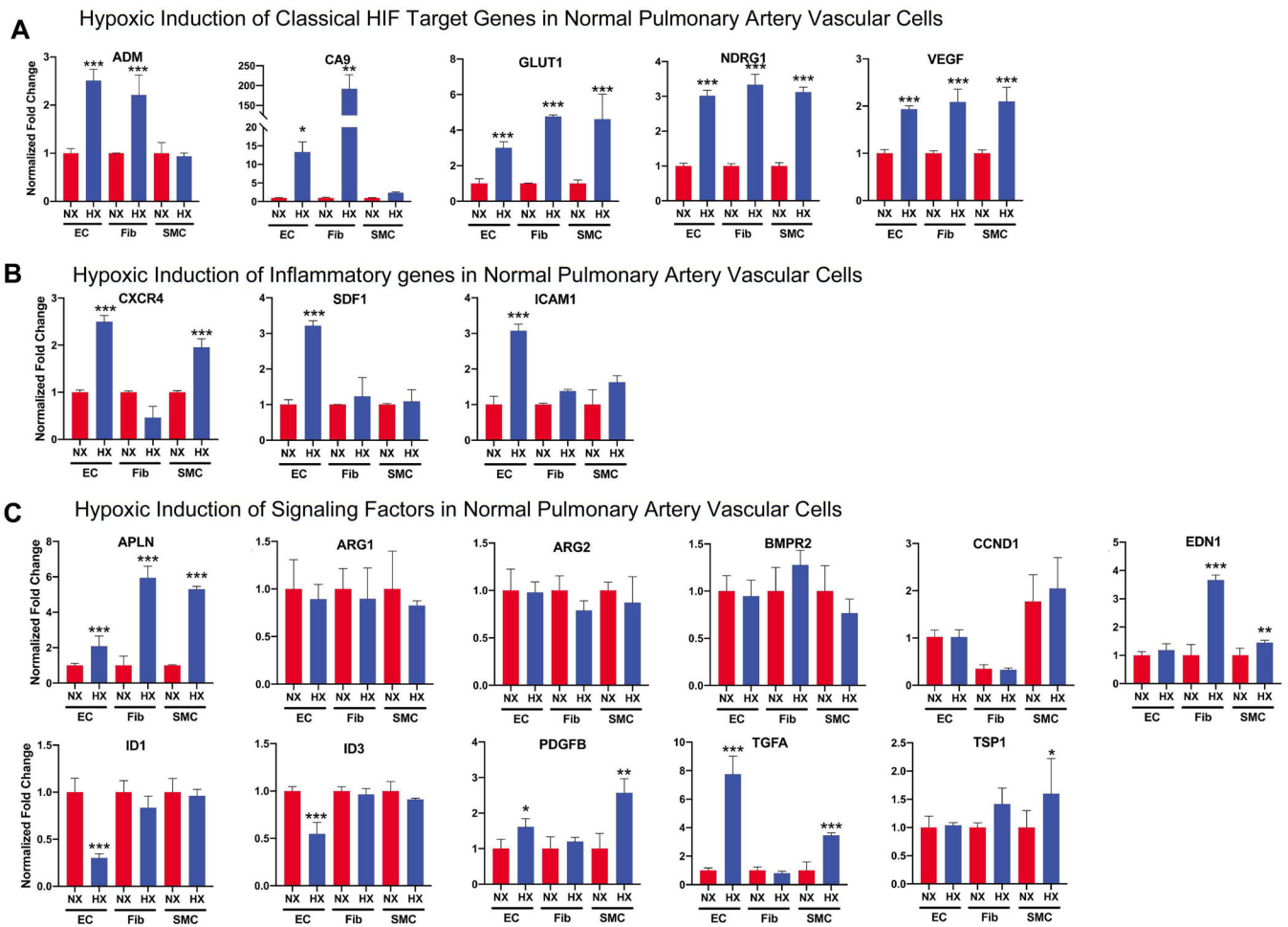


Figure 6. Normal human pulmonary artery vascular cells exhibit unique properties in response to acute hypoxia.

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.

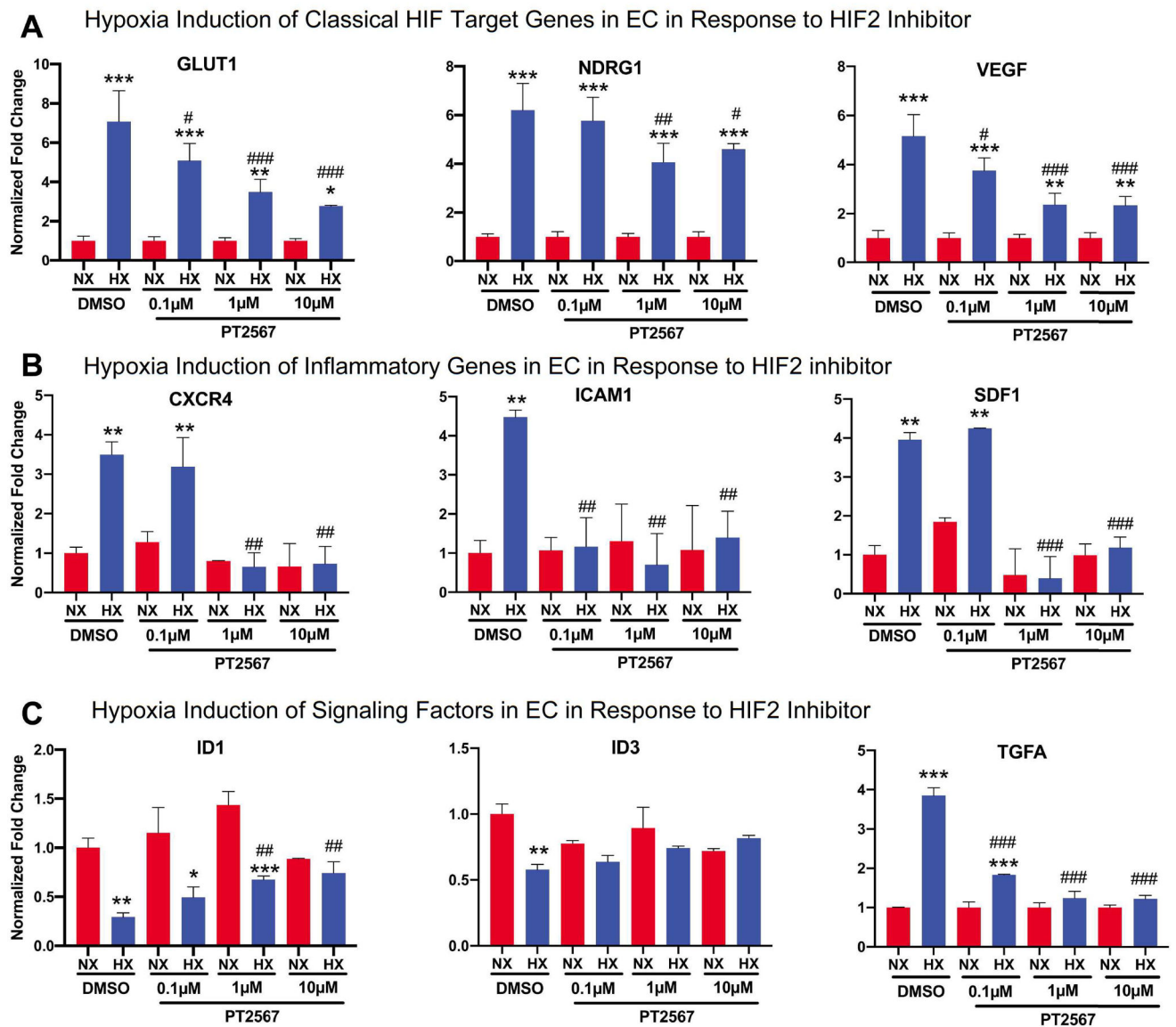


Figure 7. HIF2 inhibitor PT2567 significantly attenuates altered production of genes involved in inflammation and signaling in normal pulmonary endothelial cells in response to acute hypoxia. To determine if HIF2 activity is responsible for hypoxia-mediated gene expression changes in EC, normal human 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.

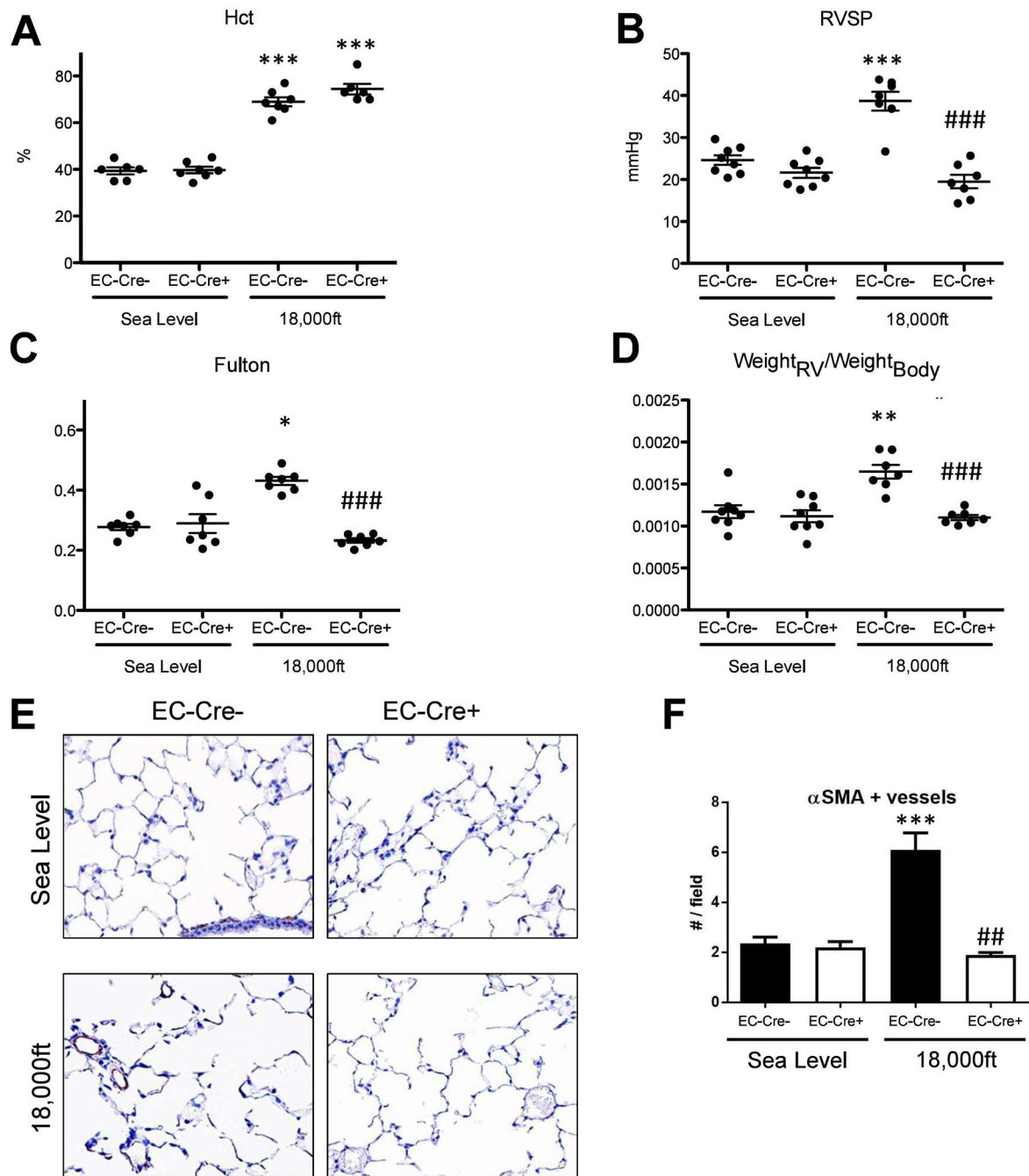


Figure 8. Hif2 α expression in endothelial cells is required for development of hypoxia-induced PH.

Endothelial Hif2 α knockout or Hif2 α 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).