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# Revisiting the role of hypoxia-inducible factors in pulmonary hypertension

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Pulmonary hypertension (PH) is a deadly condition with limited treatment options. Early studies implicated hypoxia-inducible factors (HIFs) as contributing to the development of hypoxia-induced PH. Recently, the use of cells derived from patients and transgenic animals with cell-specific deletions for various parts of the HIF system has furthered our understanding of the mechanisms by which HIFs control pulmonary vascular tone and remodeling to promote PH. Additionally, identification of HIF inhibitors further allows assessment of the potential for targeting HIFs to prevent and/or reverse PH. In this review, recent findings exploring the role of HIFs as potential mediators and therapeutic targets for PH are discussed.

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## Introduction

By definition, pulmonary hypertension (PH) is diagnosed as mean resting pulmonary arterial pressure (PAP) reaching and/or surpassing 25 mmHg and clinically portends a significant increase in morbidity and mortality. Around the turn of the 20th century, Romberg and Ayerza first recognized PH as a form of cyanosis, dyspnea, and chest pain leading to right heart failure, which was confirmed on autopsy diagnosis [1,2]. In 1935, Brenner formed the basis of understanding of PH, finding PH not to be a single pathologic entity but 'several different conditions', providing the first insight into classification of a condition that is still being perfected [2].

Following decades of clinical and animal research, the proposed classification changed in 1998 from primary PH and secondary PH to a system based on clinical diagnoses and treatment [3]. The World Health Organization now classifies PH into 5 groups [4] as described in Table 1. With common pathobiology and response to treatment, Group 1 was called pulmonary arterial hypertension (PAH). Groups 2–5 describe PH where the etiology could be best explained by left heart disease, chronic lung disease with or without hypoxemia, chronic thromboembolic pulmonary hypertension (CTEPH) and unclear multifactorial mechanisms, respectively. Perhaps because of this wide variation, the mechanisms involved in disease initiation and progression are still poorly understood. In this article, we will review recent findings exploring the role of hypoxia-inducible factors (HIFs) as potential mediators of this disease.

## Common animal models of PH

Much of the understanding of this rare disease comes from animal research. PH, being a syndrome that results from many distinct pathobiological states, cannot be completely replicated by a singular animal model. Hence, development of models that, to some extent, are reflective of different PH groups was essential to increased understanding of the molecular underpinnings of PH and emerging possibilities to target morbidity.

### Rat models

An easy to implement rat model where PH develops rapidly uses monocrotaline (MCT), a toxic alkaloid. MCT causes not only severe PH and high mortality, but also pneumotoxicity involving the airways, alveoli, and vasculature [5–7]. Hence, although widely adopted, MCT is not considered as physiologic as other models. Fawn-hooded rats, which have a platelet serotonin storage defect, develop spontaneous PH with age, which can be accelerated with exposure to mild hypoxia (reviewed in Ref. [8]). Displaying robust pulmonary vascular remodeling, these animals have been considered a potential model of human PAH, although they also develop systemic hypertension [9]. One of the best-established models to study PH is chronic hypoxia (CH), a model of Group 3 PH. In rats, exposure to CH (typically 10% $O_2$ for 1–4 weeks) causes significant pulmonary vascular remodeling, smooth muscle proliferation, decreased vascular cell apoptosis, and augmented vasoconstriction (reviewed in Ref. [8]).

The addition of an injection of the vascular endothelial growth factor (VEGF) receptor small molecule inhibitor, SU5416, to hypoxic exposure (SuHx) in rats significantly increases PAP and inflammatory infiltration and results in development of reproducible, robust pulmonary vascular

**Table 1**

**Classification of pulmonary hypertension**[a]

| |
|---|
| **Group 1—Pulmonary arterial hypertension (PAH)** |
| Idiopathic PAH |
| Heritable PAH |
| Drug and toxin induced |
| Associated with connective tissue disease; infections; portal hypertension; congenital heart diseases |
| Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomatosis |
| Persistent pulmonary hypertension of the newborn |
| **Group 2—Pulmonary hypertension due to left heart disease** |
| Left ventricular systolic dysfunction |
| Left ventricular diastolic dysfunction |
| Valvular disease |
| Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies |
| **Group 3—Pulmonary hypertension due to lung diseases and/or hypoxia** |
| Chronic obstructive pulmonary disease |
| Interstitial lung disease |
| Other pulmonary diseases with mixed restrictive and obstructive pattern |
| Sleep-disordered breathing |
| Alveolar hypoventilation disorders |
| Chronic exposure to high altitude |
| Developmental lung diseases |
| **Group 4—Chronic thromboembolic pulmonary hypertension (CTEPH)** |
| **Group 5—Pulmonary hypertension with unclear multifactorial mechanisms** |
| Hematologic disorders |
| Systemic disorders |
| Metabolic disorders |
| Others |

[a] Modified from Ref. [4].

remodeling and vaso-occlusive lesions resembling the complex neointimal lesions observed in human PAH [10–12]. The mechanisms involved in the pathogenesis of PH in this model are believed to include endothelial cell (EC) death followed by advent of an apoptosis-resistant EC population [11,12]. Importantly, PH induced by SuHx in rats is not typically reversible with return to normoxia, although there are reports of potential reversibility and presence of right ventricle (RV) failure without increase in mortality [13]. Nonetheless, the SuHx rat model has become the current 'gold standard' for studying possible mechanisms in PAH.

### Murine models

Mouse models of PH offer the advantage of readily available genetically modified strains to test the roles of specific proteins. Unfortunately, mice are typically less susceptible to MCT and CH, the latter varying across different strains (reviewed in Ref. [14]). Nonetheless, CH in mice remains a common model for Group 3 PH. The severity of PH induced can be increased by combining CH with genetic or pharmacological manipulations. For example, transgenic mice expressing a dominant-negative bone morphogenetic protein receptor type 2 (BMPR2) mutation similar to that observed in humans develop sporadic PH [15], the severity and incidence of which can be increased with hypoxic exposure [16]. Similarly, a SuHx protocol resulted in enhanced PH [17], with reported robust vascular remodeling and vaso-occlusive lesions, although the murine SuHx model has had various levels of success [18]. Importantly, repeated injections of SU5416 and continued CH exposure are required to maintain PH in the mouse SuHx model. Along the same lines, transgenic mice overexpressing the serotonin transporter (SERT), leading to increased cellular serotonin uptake, develop PH which is exaggerated in the presence of hypoxia [19]. Interestingly, only female SERT mice exhibit the PH phenotype [20], reminiscent of the higher female:male ratio of human PAH.

## Hypoxia inducible factors

Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors, consisting of α and β subunits, which bind hypoxia response elements in target genes (reviewed in Ref. [21]). The α subunit is highly inducible by hypoxia, and three different isoforms have been identified (HIF-1α, HIF-2α and HIF-3α); all of which interact with HIF-1β. Unlike the α subunits, HIF-1β, also called the aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively expressed. The α and β subunits have similar domains: N-terminus basic helix-loop-helix (bHLH) domains for DNA binding and a central region PER-ARNT-SIM (PAS) domain, which facilitates heterodimerization [22,23]. The COOH-terminal halves of HIF-1α and HIF-2α contain two transactivation domains and $O_2$-dependent degradation domains [24,25]. HIF-3α shares a similar, but less related and understood, domain [24].

In the presence of oxygen, prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIH) serve to downregulate and inactivate the α subunits [25,26]. In humans, PHDs hydroxylate two conserved proline residues to promote α subunit targeting by the von Hippel-Lindau (VHL) tumor suppressor, E3 ubiquitin ligase, resulting in rapid destruction via the ubiquitin/proteasome pathway [27]. FIH hydroxylates an asparagine residue, inactivating HIF transcriptional activity by blocking recruitment and association with the co-activator, p300 [28,29]. Under hypoxic conditions, PHD activity is decreased and subsequent degradation processes are suppressed, allowing rapid stabilization of the α subunits, which then translocate into the nucleus and form a transcriptionally active complex with HIF-1β.

The HIF signaling cascade plays a critical role in physiological and pathobiological processes, including angiogenesis, glycolysis, apoptosis, erythropoiesis, cellular proliferation, inflammation, embryonic development, ischemic cardiovascular disease, wound healing, and cancer (reviewed in Refs. [30,31]). In general, HIF-1 and HIF-2 play equally important roles in oxygen sensing,

homeostasis, development, physiology, and pathobiology and target overlapping, but in some instances distinct, genes [30,31]. Complete HIF-1α or HIF-1β deficiency results in developmental cardiovascular defects and embryonic lethality [32,33], whereas HIF-2α deficiency causes fetal death in approximately 50% of embryos, with survivors exhibiting impaired lung development and neonatal lethality [34]. In contrast, animals heterozygous for HIF-1α or HIF-2α develop normally with no apparent dysfunction in the absence of stressors [35,36].

## Role of HIF in PH

Given the use of CH as a stimulus for PH, it is not surprising that HIFs have been explored as potential mediators of PH. With some exceptions, studies have generally found HIFs to be upregulated in PH, although the conditions under which each HIF contributes, and in which cell types, are still being identified.

### Expression of HIFs in PH

Evidence for alterations in the HIF system during PH has come on multiple levels. In whole lung tissue, HIF-1α protein levels were increased in idiopathic pulmonary fibrosis (IPF) patients with PH compared to IPF alone [37•], and in whole lung tissue from a lamb model with persistent PH of the newborn [38]. Attempts to localize which HIF might be upregulated, and in which vascular cell type, have yielded variable results. Reporter experiments demonstrated that HIF promoter activity was increased in pulmonary arterial smooth muscle cells (PASMCs) from PAH patients [39••]. Consistent with these results, immunohistochemical analysis revealed increased HIF-1α protein levels in PASMCs in IPF patients with PH [37•] and in the media of small diameter PAs from patients with PAH [40,41,42••,43•]. HIF-1α was also upregulated in cultured PASMCs from Fawn hooded rats [41], PAH patients [40] and CH rats [44]. However, at least one study reported a reduction in HIF-1α in PAH patient PASMCs [45•].

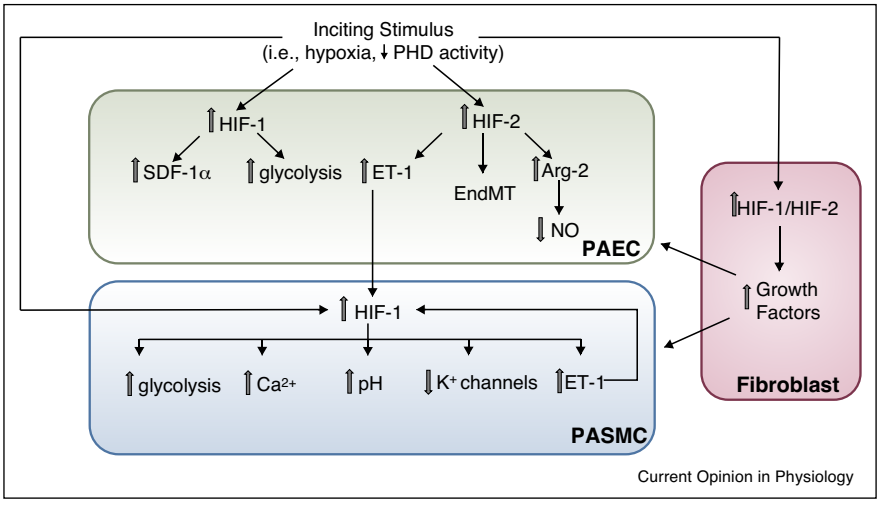
While HIF-1 is ubiquitously expressed, HIF-2 is highly expressed in ECs. Several studies demonstrated increased HIF-2α levels in PAH ECs either *in vivo* [42••] or in culture [42••,43•]. In some studies, HIF-1α was also upregulated in the intima of vessels [37•,40] and in cultured ECs from PH patients [45•,46]. Surprisingly, in at least one of these studies, HIF-2 in ECs was not increased [45•]. These discrepancies, along with data reporting increased HIF-2α in PASMCs in small diameter PAs from PAH patients [47] and in PASMCs from CH rats [44] or that HIF-1α was decreased in PASMCs from PAH patients [45•], paint a confusing picture and raise the question as to how well cultured cells replicate *in vivo* pathology and whether etiology of disease results in variations in cellular expression of HIFs. It is unclear whether and how location from where the cells were derived (proximal versus distal lung), culture conditions (full versus low serum media, passage number, etc), stage or etiology of disease, sex and/or treatment regimen might impact HIF-1/2 levels. Another issue that may contribute to varied results is that, given the rare nature of the disease, all studies using patient tissues had small sample sizes. As biorepositories of patient samples expand, data with larger 'n' may help resolve some of the wide variations observed with patient samples and shed additional light on the reasons for these varied findings.

### HIF loss-of-function and PH

The first direct evidence for a role for HIFs came from studies using mice with partial deficiency for either HIF-1α [36] or HIF-2α [35], both of which exhibited reduced PH. However, in these early studies, the cell-specific roles of HIF-1 and HIF-2 could not be determined. Subsequent studies have tried to answer this question, with varied results. An initial study found targeted deletion of smooth muscle HIF-1α using a constitutive SM22α-driven Cre did not protect mice from development of CH-induced PH; in fact RV systolic pressure (RVSP) increased slightly [48]. That both male and female mice were used may complicate interpretations given that females are often protected from hypoxia-induced PH. A subsequent study using male mice with inducible SMC-targeted deletion of HIF-1α using SMM-driven Cre found reductions in both PH and vascular remodeling [49]. Surprisingly, RV hypertrophy was not reduced, perhaps indicating a HIF-1-independent effect of hypoxia on the RV. Whether the differences between these studies reflects inducible versus constitutive deletion, an influence of sex, or the driver used to produce Cre expression remains unknown. Interestingly, other studies showed that EC-specific deletion of HIF-2α, but not HIF-1α, attenuated CH-induced PH [50•,51•]. These studies would appear to indicate that both HIF-1 and HIF-2, perhaps acting in different cell types, contribute to hypoxia-induced PH (Figure 1).

Additional evidence for a role of HIFs in PH comes from studies in populations living under hypoxic conditions at high altitude. In acclimatized individuals, mutations in HIF pathway genes are associated with lack of hypoxia-induced PH (reviewed in Ref. [52]). Identified mutations include variants in *Phd3* [53] in Andeans, in *Arnt* in Ethiopians [54] and in *Epas1* (encoding HIF-2α) and *Egln1* (encoding PHD2) in Tibetans [55–59]. While the functional consequence of most of these variants has not been fully explored, the Tibetan *Engl1* mutation appears to result in gain-of-function for PHD2 activity [59], suggesting that adaptation was associated with suppression of HIFs.

**Figure 1**




Schematic providing an overview of some of the mechanisms by which HIFs promote pulmonary hypertension. Increased HIF-1 levels in pulmonary arterial endothelial cells (PAECs) have been linked to increased production of stromal-derived factor 1α (SDF-1α), the secretion of which can recruit bone marrow progenitor and immune cells, and metabolic derangements leading to increased glycolysis. Increased HIF-2 levels in PAECs enhance production of endothelin 1 (ET-1), promote endothelial-to-mesenchymal transition (EndMT) and induce expression of arginase 2 (Arg 2), which in turn reduces nitric oxide (NO) production. ET-1 can increase HIF-1 levels in pulmonary arterial smooth muscle cells (PASMCs). In PASMCs, upregulation of HIF-1 leads to mitochondrial dysregulation and increased glycolysis, increased cytosolic $Ca^{2+}$ levels, an alkaline shift in pH and downregulation of $K^+$ channels. Production of ET-1 in PASMCs results in a feed-forward loop to further enhance HIF-1 levels. Finally, in fibroblasts, increased HIF-1/HIF-2 levels lead to the production of growth factors which can act on both PAECs and PASMCs.

## HIF gain-of-function and PH

While loss of function experiments proved a role for HIF-1 and HIF-2 in hypoxia-induced PH, additional support has also been found in gain-of-function experiments. For example, in humans with a mutation in VHL leading to Chuvash disease, increased HIF levels [60] are associated with polycythemia, PH, and upregulated HIF-dependent gene expression [61]. A patient with a different loss of function VHL mutation was also reported to have severe PAH [62]. Consistent with these reports, mice with a constitutive VHL mutation mimicking Chuvash disease also exhibited robust increases in PAP, RV wall thickness and pulmonary vascular remodeling [43•,63]. In addition to PH, these animals also exhibited pulmonary edema, immune cell infiltration and fibrosis associated with higher lung levels of HIF-2α but not HIF-1α. Heterozygosity for HIF-2α attenuated, but did not completely prevent the increase in PAP [43•,63], suggesting a more sensitive role for HIF-2 in this model. Similarly, variants in *Epas1* that likely confer gain-of-function for HIF-2 were associated with PH susceptibility in cattle living at altitude [64].

Recently, multiple labs reported that mice with EC-specific deletion of PHD2, and thus upregulated endothelial HIF, develop severe PH with obliterative lesions [43•,51•,65••]. Interestingly, in contrast to hypoxic models, PH in these mice appears to be driven primarily by HIF-2 as mice with HIF-2α heterozygosity or EC-specific deletion were protected [51•]. Individuals [66] or mice [67] with HIF-2 gain-of-function mutations also have elevated resting systolic PAP, further demonstrating that overexpression of HIF-2 can lead to PH.

Taken as a whole, these data clearly support roles for both HIF-1 and HIF-2 in the development of PH, although which isoform, and in which cell type, may depend on the inciting stimulus. For example, during hypoxia HIF-2 appears to play a more prominent role in EC, while HIF-1 may be more important in PASMC. A potential explanation could stem from studies showing that HIF-1 in PASMC can be upregulated by ET-1 [68]. Of note, ET-1 activates STAT3, a known upstream regulator of HIF-1 [69]. Whether HIF-2 upregulates STAT3 in ECs or PASMCs, as it does in other cell types [70], is not clear. Moreover, the hypoxia-induced increase in circulating ET-1 levels was absent in HIF-2α heterozygotes [35]. Thus, it is possible that under conditions of hypoxia, induction of HIF-2 in ECs increases production of ET-1 [51•], leading to subsequent upregulation of HIF-1 in SMCs. In this case, both HIFs could play a necessary role, albeit in different cell types. Another possibility could relate to temporal differences in the roles of HIF-1/2. In mice partially deficient for HIF-1α, hypoxic PH was attenuated at three weeks but caught up to wild-type animals by six weeks [36], perhaps reflecting either a

delayed response from residual HIF-1 or an early role for HIF-1. Whether HIF-2α heterozygotes also catch up is unknown. Finally, while both HIF-1 and HIF-2 appear to play roles in hypoxic models, PH in mice with forced HIF expression due to genetic mutations/deletions in the degradation pathway appears to specifically require endothelial HIF-2α [51•,65••]. These differences may suggest that the role of HIFs may vary depending on inciting cause of PH.

### Mechanisms by which HIFs promote PH

Despite some variability, it would appear that the majority of studies indicate that HIFs are increased in PH. In both ECs and PASMCs from PH patients or models, increased HIF levels are associated with mitochondria dysfunction and metabolic abnormalities, which have in turn been linked to increased cellular migration, proliferation and apoptotic resistance [40,41,46,71]. Indeed, HIF-1α overexpression increased proliferation of PASMCs, whereas HIF-2α more robustly increased proliferation of ECs [72]. In PASMCs, pathways modulated by HIF-1 and controlling cell proliferation and metabolism include mitochondrial function [40,41], downregulation of $K^+$ channels [41,73,74] and upregulation of transient receptor potential channels and $Na^+/H^+$ exchanger 1, leading to elevated $[Ca^{2+}]_i$ and pH, respectively [75–77].

In addition to direct effects of HIFs on PASMCs and EC function, paracrine effects may also play a role. For example, in PAH patients, circulating levels of HIF-regulated factors, including hepatocyte growth factor and SDF-1α, are increased, corresponding to increased production from ECs [78], which may serve to promote immune cell recruitment to the pulmonary vasculature. In patients with PH related to IPF, HIF-2 appeared to be increased primarily in peripheral part of the vessel, that is, adventitial fibroblasts [79]. Induction of HIF-1 [80] or HIF-2 [81] by hypoxia in fibroblasts increases production of factors that stimulate PASMC growth [81] or migration [81]. These changes are likely to contribute to the vascular remodeling and enhanced contractile responses that result in elevated PAP.

## Targeting HIFs in PH

On the basis of the data presented above, it would appear that there is considerable potential benefit for targeting the HIF system in PH. Initial studies exploring HIF inhibition have been encouraging. For example, digoxin, which inhibits HIF-1 transcriptional activity by preventing HIF-1α protein accumulation [82], attenuated hypoxic induction of HIF-1 target genes in lungs from chronically hypoxic animals and in PASMCs exposed to hypoxia *ex vivo* and prevented PH in mice exposed to CH [83]. PH was also reduced when digoxin treatment began after PH was established. Similar beneficial effects were observed when HIFs were inhibited with acriflavine in CH rats [83]. These results suggest that targeting HIFs might prevent or slow the changes in pulmonary vascular pressure and remodeling in PH.

Recently, targeting HIF-2 using the small molecule inhibitor, C76, was shown to be effective in reducing PH in several models [42••], including in mice with forced expression of HIFs in ECs. In stark contrast to most PH models, significant mortality (50%) was observed in this model, presumably due to right heart failure. C76 treatment not only reduced PH and preserved RV function, but also had an impressive effect on survival. In these studies, HIF-2 inhibition also attenuated PH in SuHx and MCT rats when given after PH was established, suggesting a potential for therapeutic approaches. Importantly, short-term HIF-2 inhibition appeared to improve RV hemodynamics, alleviating concerns that HIF inhibition could impair adaptive cardiac responses to pressure overload [84].

## Conclusion

It is evident that in the nearly 20 years since the first studies demonstrated a role for HIFs in PH the exact role HIFs play has emerged as a complicated picture. Temporal, cell and stimulus specificity likely contribute to the varied findings reported. A better understanding of the factors and conditions under which HIF-1 and HIF-2 contribute to processes involved in disease progression is clearly needed. As increasing information becomes available, HIF inhibitors are more and more likely to be attractive candidates and are worth further study. An important question that will need to be addressed will be whether targeting either HIF alone, or both HIFs together, is a feasible approach. In a disease with limited treatments, careful future clinical trials will be needed to elucidate whether, and under what conditions and in which patients, HIF inhibitors might be an option for therapeutically treating, or even curing, PH.

## Conflict of interest statement

Nothing declared.

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