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Review

Role and regulation of prolyl hydroxylase domain proteins

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Oxygen-dependent hydroxylation of hypoxia-inducible factor (HIF)-α subunits by prolyl hydroxylase domain (PHD) proteins signals their polyubiquitination and proteasomal degradation, and plays a critical role in regulating HIF abundance and oxygen homeostasis. While oxygen concentration plays a major role in determining the efficiency of PHD-catalyzed hydroxylation reactions, many other environmental and intracellular factors also significantly modulate PHD activities. In addition, PHDs may also employ hydroxylase-independent mechanisms to modify HIF activity. Interestingly, while PHDs regulate HIF-α protein stability, PHD2 and PHD3 themselves are subject to feedback upregulation by HIFs. Functionally, different PHD isoforms may differentially contribute to specific pathophysiological processes, including angiogenesis, erythropoiesis, tumorigenesis, and cell growth, differentiation and survival. Because of diverse roles of PHDs in many different processes, loss of PHD expression or function triggers multi-faceted pathophysiological changes as has been shown in mice lacking different PHD isoforms. Future investigations are needed to explore *in vivo* specificity of PHDs over different HIF-α subunits and differential roles of PHD isoforms in different biological processes.
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In response to hypoxia, metazoans from worms to humans increase glycolysis to compensate for energy loss due to reduced oxidative phosphorylation, and upregulate erythropoiesis and angiogenesis to achieve more efficient oxygen utilization. Central to this adaptive response is the mechanism that uses oxygen deficiency as a signal to activate the transcription of genes important for these processes. The key to this activation mechanism is the rapid accumulation of hypoxia-inducible factor (HIF)-α under hypoxia.[1,2] In essentially all cell types examined so far, HIF-α subunits undergo oxygen-dependent hydroxylation on specific proline residues. Hydroxylated HIF-α subunits are recognized by the von Hippel–Lindau (VHL) protein of the E3 ubiquitin ligase complex, and are rapidly destroyed via the polyubiquitination/proteasomal degradation pathway.[3–7] As intracellular O_2 concentration is reduced, non-hydroxylated HIF-α subunits accumulate and form functional transcription factors in the nucleus by heterodimerization with the constitutively expressed HIF-1β subunit.[1]

HIF-prolyl hydroxylases (HPHs),[6] also referred to as prolyl hydroxylase domain (PHD) proteins,[7] form an evolutionarily conserved subfamily of dioxygenases that uses oxygen and 2-oxoglutarate (2-OG) as co-substrates, and iron and ascorbate as cofactors.[6–8] While these enzymes are most intensively studied in animals especially mammalian cells, a similar hydroxylase has also been described in the plant *Dictyostelium*, where it hydroxylates SKP1, a homolog of the

FBOX component of the E3 ubiquitin ligase.[9] In addition, enzymes with similar catalytic centers may also exist in some prokaryotes.[10] While *Drosophila* and *Caenorhabditis elegans* each has a single family member, known as dHPH in *Drosophila*[6] and *eg*g *l*aying *n*ine (EGLN or EGL-9) in *C. elegans* (for its essential egg-laying function),[7] mammals have four members belonging to this subfamily, including PHD1/EGLN2/HPH3, PHD2/EGLN1/HPH2, PHD3/EGLN3/HPH1 and a recently characterized protein named as P4H-TM.[6,7,11] Several other names have also been used for mammalian homologs, including SM-20 for rat PHD3[7] and HIF-P4H1, HIF-P4H2 and HIF-P4H3 to indicate that hydroxylation occurs on position 4 of proline residues.[12] Given that the term 'PHD proteins' both reflects the functionality as hydroxylases and avoids committing them as being HIF specific (because non-HIF substrates also exist[13]), we will refer to mammalian homologs as PHD1, PHD2 and PHD3, however, keeping the name P4H-TM[11] for the fourth member of the subfamily due to the fact that other names have not been used in the literature.

While O_2 concentration is the most ubiquitous factor controlling PHD-catalyzed hydroxylation reactions, there is now strong evidence that various other parameters may also come into play.[14] Furthermore, hydroxylation of non-HIF substrates and hydroxylase-independent functions have also been reported for PHDs or their plant homologs.[9,13,15,16] Thus, in addition to serving as oxygen sensors, PHDs may

potentially have other functions as well. In this review, we provide a summary and analysis of current literature on PHDs and discuss their roles in regulating oxygen homeostasis as well as other pathophysiological processes.

Regulation of PHD-Catalyzed Hydroxylation Reactions

Both full-length PHD1 and PHD2 have over 400 (407 and 426 in humans, respectively) amino-acid residues and share a well-conserved hydroxylase domain at their C-terminal halves ($\sim$55% identity within hydroxylase domains), whereas the N-terminal halves are more divergent and have poorly characterized functions.[7] The much shorter PHD3 (239 amino-acid residues in humans) contains the hydroxylase domain but only a short stretch of the divergent N-terminal sequence. Since basic enzymatic mechanisms of PHD hydroxylases have been reviewed extensively,[2,8,17] here we focus on how the hydroxylase activity is regulated (Figure 1).

Oxygen. Oxygen is a key substrate for hydroxylation. All three PHDs have K_m values for oxygen within the range of 230–250 μM, slightly above the oxygen concentration in aqueous solutions saturated by ambient room air.[12] Since intracellular oxygen concentrations are typically below these values, high K_m values ensure the dependence of hydroxylase activity on oxygen when all other substrates and cofactors are present at adequate levels.

Cells cultured under severe hypoxia (0.5–2% oxygen) almost invariably accumulate HIF-1α; however, recent studies found that such accumulation is dependent on functional mitochondria.[14,18] Respiratory inhibitors, such as nitric oxide

(NO) which inactivates cytochrome *c* oxidase, causes destabilization of HIF-1α even under hypoxia.[14,18] One theory proposes that functional mitochondria rapidly consumes oxygen through oxidative phosphorylation, thus diminishing O_2 concentration in the cytosol[18] (Figure 1). When mitochondrial oxygen consumption is inhibited, oxygen is 'redistributed', resulting in an increase in cytosolic oxygen concentration. Under normoxia, however, cytosolic oxygen levels may remain sufficiently high for active hydroxylation in spite of rapid consumption by mitochondria. This interpretation is consistent with the finding that mitochondrial inhibitors do not substantially increase PHD activities under normoxia.[14]

In addition to oxygen-dependent prolyl hydroxylation mediated by PHDs, HIF-1α is subject to oxygen-dependent asparaginyl hydroxylation by factor inhibiting HIF (FIH).[19,20] FIH is also an iron-dependent dioxygenase and hydroxylates Asn 851 located within the C-terminal transactivation domain of HIF-1α. Hydroxylation at Asn 851 inactivates HIF-1α by preventing its interaction with CBP/p300, an essential coactivator for HIF-dependent transcription.[19,20]

Intracellular Fe(II) concentration. NO may inhibit PHD activity by chelating Fe(II) or other mechanisms in addition to the previously mentioned role in promoting PHD activity by oxygen redistribution[21,22] (Figure 2). The net outcome may depend on which effect happens to dominate and is more easily observed under specific experimental conditions. For example, while high concentrations of NO ($>1\,\mu$M) inhibit PHD activity, the resultant stabilization of HIF-1α is easily detectable only under normoxia, whereas under hypoxia such an effect is masked by hypoxia-induced HIF-α accumulation. Lower NO concentrations ($<0.4\,\mu$M) are insufficient to inhibit PHD activities, thus the ability to promote PHD activity (and hence destabilizing HIF-α) plays a major role. However, such
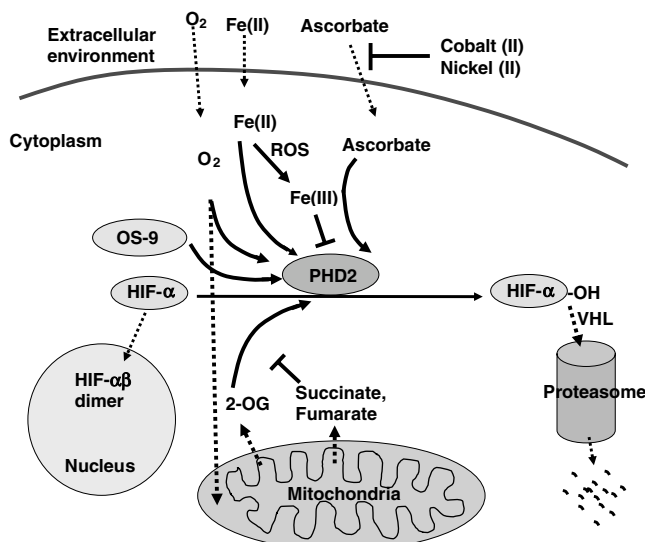

Figure 1 Schematic illustration of regulatory mechanisms of PHD hydroxylase activities. Regulation of hydroxylase activity by small molecules and protein factors are indicated, but regulation at the level of PHD expression is not shown. Dashed arrows indicate movements of molecules in the direction of the arrows, but solid arrows indicate functions in favor of the indicated direction. For example, ascorbate is indicated by dashed arrow from the extracellular environment into the cytoplasm to show that it is imported from outside, but the solid arrow between ascorbate and PHD2, following the direction of HIF-1α to HIF-1α-OH reaction, indicates that ascorbate supports this reaction. On the other hand, a 'T'-shaped symbol indicates the interference of certain functions. An example of this is the interference of the role of 2-OG by succinate and fumarate

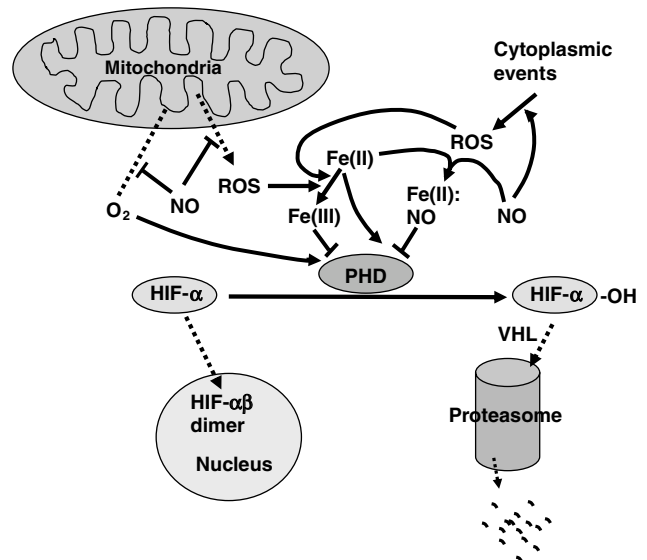**Figure 2** Regulation of PHD hydroxylase activities by NO and ROS. These molecules may have multiple points of interaction with PHD regulatory mechanisms or originate from different sources, which is consistent with the complexity of the effects of NO and ROS revealed in various studies. Merging of arrows originating from Fe(II) and NO indicate binding between Fe(II) and NO; other symbols are the same as explained in Figure 1

a role is prominent only under hypoxia, whereas under normoxia the contribution of NO-induced increase in PHD activity may be negligible compared to PHD activation by high concentrations of oxygen.[18]

Reactive oxygen species (ROS) may inhibit PHDs by chelating and oxidizing PHD bound Fe(II) to Fe(III)[14,23] (Figure 2). Mitochondrial ROS production is associated with oxidative phosphorylation, but interestingly ROS production increases with reduced availability of oxygen due to stress.[24,25] Thus, oxidative phosphorylation under hypoxia may inhibit PHD activity by abundant ROS production.[14] In this regard, NO or other mitochondria inhibitors may activate PHD activity by preventing ROS production in mitochondria,[14] a mechanism that is not mutually exclusive with the oxygen sink theory[18] (Figure 2).

Of note, one study found that defective oxidative phosphorylation under hypoxia (hence less ROS) did not compromise hypoxia-induced HIF-1α stabilization,[26] suggesting that ROS effects on PHD activity are potentially complicated. On the other hand, ROS is not only generated in mitochondria but also by various cytoplasmic activities (Figure 2). For example, $JunD^{-/-}$ mutation leads to elevated RAS signaling and ROS production, which leads to decreased PHD activities and HIF-1α stabilization.[23] Under conditions where cytosolic ROS production is significantly elevated, a reduction in mitochondrial ROS production may not have much noticeable effect on total ROS concentration and therefore fails to further inhibit PHD activity. This interpretation may help explaining some discrepancies in the literature.

Some environmental factors, such as nickel (II) and cobalt (II), can inhibit PHD activity and cause HIF-α stabilization.[27] Interestingly, exposure of cells to these ions greatly reduced intracellular ascorbate concentrations. However, PHD activities could be restored by supplying higher concentrations of ascorbate to culture media, suggesting that nickel (II) and cobalt (II) block the entry of ascorbate into the cell[27] (Figure 1). These findings suggest the importance of ascorbate in PHD activities, likely by reducing PHD-bound Fe(III) back to Fe(II).

Competitive inhibition by 2-OG analogs. 2-Oxoglutarate, an intermediate of the tricarboxylic acid (TCA) cycle, is an essential co-substrate for PHDs due to its role in Fe(II) coordination in the catalytic center.[7] However, mitochondria also generate inhibitory TCA cycle intermediates, including succinate and fumarate which compete against 2-OG for binding to the active site[28] (Figure 1). Under normal circumstances, these molecules probably do not significantly interfere with PHD function due to the fact that their cytosolic concentrations are very low. However, mitochondrial defects or manipulations that lead to significant cytosolic accumulation of these intermediates are associated with abundant HIF-1α accumulation due to competitive inhibition of PHD activities.[14,28–30] Another TCA cycle intermediate oxaloacetate and the glycolysis product pyruvate were also reported to inhibit PHDs in some experimental systems but not in others, suggesting that their effects may be complicated and are influenced significantly by experimental conditions.[14,31]

Prolyl substrate specificity. Hydroxylation occurs on the fourth position on P402 and P564 in human HIF-1α (or at similar positions in HIF-2α) within the so-called oxygen-dependent degradation domains (ODDs).[32] The ODD domain was originally identified as a region containing about 200 amino-acid residues that overlap with the transactivation domain;[32] however, subsequent studies have shown that two independent subregions, each containing an LXXLAP core with short flanking sequences, were hydroxylated at the proline residue of the LXXLAP core.[33] While both proline residues are located within the C-terminal half of HIF-α, each LXXLAP core along with its short flanking sequences is termed N-ODD or C-ODD, respectively, depending on their relative proximity to the N and C termini.

All PHDs are more active on the C-ODD than N-ODD, and in particular PHD3 is virtually inactive on N-ODD.[12] In addition, PHD2 hydroxylates HIF-2α N-ODD less efficiently than HIF-1α N-ODD.[12,34] Detailed mechanisms of ODD selectivity are not known. In PHD2, however, several antiparallel β-sheets, formed by the sequence approximately between positions 197 and 380, are likely involved in ODD interaction. Patches of hydrophobic amino-acid residues in this region are likely the most important elements in mediating ODD substrate recognition.[10]

Surprisingly, besides the proline residue itself, other conserved residues of the LXXLAP core are not essential for substrate recognition.[35,36] Gal4-ODD fusion proteins carrying various mutations in the core sequence (except for the proline residue itself) were hydroxylated almost as efficiently as wild-type controls.[35] However, mutations beyond the conserved core significantly affected hydroxylation efficiency.[37] Interestingly, when positions of N-ODD and C-ODD were exchanged, the efficiencies of their hydroxylation by PHD1 and PHD2 were also changed accordingly. Nevertheless, PHD3 remained exclusively active on the original C-ODD even after it was moved to the N-ODD position.[38]

In cultured cells, all three PHDs contribute to the regulation of both HIF-1α and HIF-2α, but preferences do exist. PHD1 and PHD3 are more active on HIF-2α than HIF-1α, whereas PHD2 hydroxylates HIF-1α more efficiently.[34] Consistent with these cell culture-based studies, PHD2 deficiency in mouse liver and kidney tissues was associated with the accumulation of nuclear HIF-1α but not HIF-2α.[39] In contrast, PHD1/PHD3 double deficiency led to hepatic accumulation of HIF-2α but not HIF-1α.[40] Although relative abundance of different PHDs in a particular cell type may contribute to differential hydroxylation, preferences still exist even in cells expressing comparable levels of different PHDs. Furthermore, even though PHD3 is the most efficient in C-ODD hydroxylation, it does not compensate for the loss of PHD2 in destabilizing HIF-1α.[34] At the meantime, the lack of PHD3 hydroxylase activity toward the N-ODD does not explain why it cannot replace PHD2 because N-ODD hydroxylation contributes only minimally to HIF-1α degradation.[37]

These complexities indicate that selectivity of HIF-α hydroxylation by different PHDs may go beyond ODD sequence motifs or even hydroxylase activity alone. In this regard, it is important to note that other proteins do exist that modify hydroxylation activity by means of protein–protein interactions. For example, OS-9 brings HIF-1α and PHDs into a multi-protein complex and promotes hydroxylation.[41]

Regulation of PHD Availability

PHD2 was initially found to be the most critical hydroxylase in a large variety of cultured cells;[42] however, subsequent analyses found that one important reason was its more abundant expression than other isoforms.[34] When PHD1 and PHD3 expression levels were increased by transfection, the importance of these isoforms was also revealed, especially in the hydroxylation of HIF-2α.[34] These studies illustrate that regulation of PHD abundance plays a crucial role in oxygen sensing.

Tissue and intracellular distribution. At the RNA level, all three *PHD*s are widely distributed among different organs, although *PHD1* is highest in testes whereas *PHD3* is the highest in the heart.[43] At the protein level, however, PHD2 is the most abundant in all mouse organs examined,[40] which is consistent with other studies demonstrating PHD2 being the most abundant isoform in different cell lines.[34,42]

Intracellular localization patterns of three PHDs have been studied. While initial analysis using PHD-GFP fusion proteins indicated differential intracellular localization of different PHDs,[44] a subsequent study using monoclonal antibody indicated that all endogenous PHDs were mostly located in the cytoplasm.[45] Interestingly, P4H-TM, which is related to PHDs, is anchored in the ER membrane with its catalytic domain located in the lumen rather than cytosol.[11] In spite of such an orientation, the enzyme hydroxylates HIF-1α and triggers HIF-1α degradation.

Transcriptional regulation. PHD2 and PHD3 genes contain hypoxia response elements[46,47] and are induced by HIF-1α under hypoxia[46,48–50] (Figure 3). Not surprisingly, inhibition of PHDs by NO or other molecules may also result in feedback upregulation of PHDs due to HIF-α accumulation.[51] Besides serving as a negative feedback mechanism under hypoxia, HIF-induced PHD expression may also ensure rapid removal of HIF-α after reoxygenation.[49,52] Feedback loops may exist at different levels of hypoxia signaling. For example, while PHD2 expression is induced by hypoxia, TGF-β1, which is also induced by hypoxia, inhibits *PHD2* transcription through Smad-mediated signaling.[53] Thus, TGF-β1 seems to counter the effect of HIF-induced upregulation of *PHD2*.

Regulation of stability. While the major function of PHDs is to regulate the stability of HIF-α, PHD1 and PHD3 themselves are targeted for polyubiquitination and proteasome-mediated degradation by Siah1α/2[54] (Figure 3). Interestingly, Siah2 itself is strongly induced by hypoxia, which contributes to accelerated PHD degradation, especially that of PHD3.[54] Thus, while PHD3 transcription is induced by hypoxia, its protein degradation is also accelerated.[54] Even though Siah1α/2 do not regulate PHD2 stability, FKBP38, a peptidyl prolyl cis-trans isomerase, plays a moderate role in destabilizing PHD2 (Figure 3). Interaction between the N-termini of FKBP38 and PHD2 mediates PHD2 destabilization but the process is independent of isomerase activity.[55]

Hydroxylase-Independent or HIF-Independent Functions

PHDs may also inhibit HIF-1α transcriptional activity in addition to reducing its abundance. A complex formed between PHD2 and ING4, a tumor repressor protein, inhibits
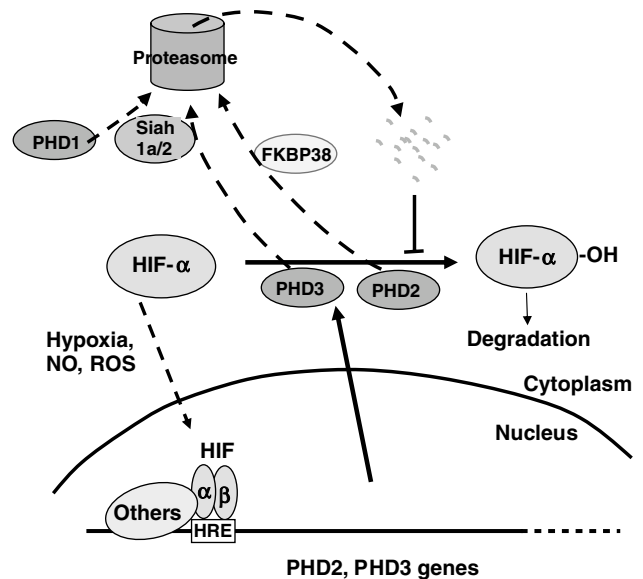

Figure 3 Regulation of PHD expression and abundance. *PHD2* and *PHD3* genes contain hypoxia response element (HRE) and are transcriptionally upregulated by HIF. Hypoxia or inhibitors that prevent HIF-α degradation promote HIF-α nuclear translocation and heterodimerization with HIF-1β. This feedback upregulation results in increased PHD2 and PHD3 abundance, which suppresses further accumulation of HIF-α. PHDs are also subject to proteasomal degradation, assisted by Siah1a/2 or FKBP38 as indicated

HIF-α-mediated expression of angiogenic cytokines and consequently suppresses tumor angiogenesis[15] (Figure 4). In support of a hydroxylase-independent role, we also found that overexpression of hydroxylase-deficient PHD2 mutant diminished the stimulating effect of hypoxia on the proliferation of cultured endothelial cells, without altering HIF-1α abundance.[16] In another study, overexpression of PHD2 in a VHL-deficient cell line also inhibited HIF-1α activity although HIF-1α abundance remained the same.[52]

Map organizer 1 (Morg1), a WD-repeat protein, serves as a scaffold protein for the interaction between HIF-1α and the divergent N-terminal sequence of PHD3.[56] This interaction reduces HIF-1α target gene expression without decreasing HIF-1α abundance,[56] suggesting possible involvement of a hydroxylase-independent mechanism. However, it is also likely that formation of a complex between Morg1, PHD3 and HIF-1α might instead prevent hydroxylated HIF-1α from entering the degradation pathway.

Hydroxylase-dependent but HIF-α-independent functions have also been described for PHD1 and PHD2 in regulating the transcriptional activity of NFκB[13] (Figure 4). The non-HIF hydroxylation substrate involved is IKKβ, a kinase that in its non-hydroxylated form phosphorylates inhibitory factor IκBα. Phosphorylated IκBα dissociates from NFκB, resulting in the activation of NFκB transcriptional activity. Under normoxia, IKKβ is hydroxylated by PHD1 or PHD2, and such a modification inhibits its kinase activity. Thus, hydroxylated IKKβ fails to trigger dissociation of the inhibitory IκBα from NFκB. When PHD1 or PHD2 is knocked down by siRNA, or inhibited by hypoxia, IKKβ hydroxylation is inhibited, thus allowing its kinase activity to phosphorylate IκBα, causing its dissociation from NFκB.
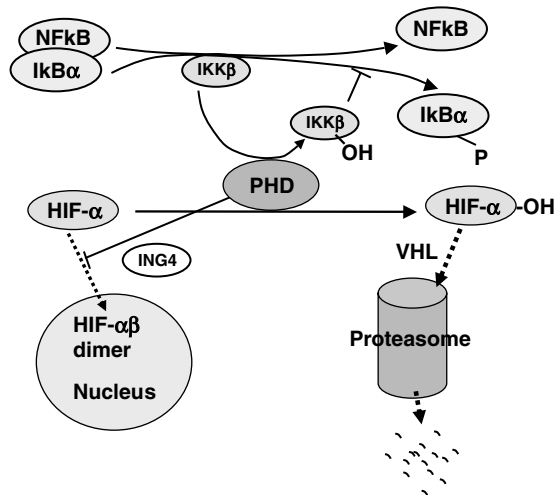
Figure 4 PHDs employ multiple mechanisms to regulate oxygen homeostasis, including regulation of HIF-α abundance, activity and NFκB activity. -P and -OH indicate phosphorylation and hydroxylation modifications, respectively. Dashed lines with arrows at the end indicate molecular movements. Labeled ovals promote a process if placed along a solid line with arrow; blunting with a short perpendicular line at the end indicates inactivity or negative regulation
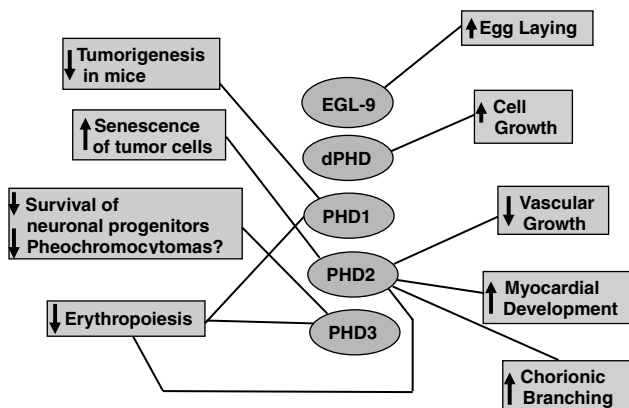

Figure 5 Currently identified biological functions of PHDs. *In vivo* processes regulated by PHDs are indicated in text boxes, with upward arrows indicating promotion of a process and downward arrows indicate suppression of a process

Biological Functions of PHDs

Various functions have been found for different PHDs, which are summarized in Figure 5. In *C. elegans*, loss of EGL-9, the prototype of PHDs, is associated with significantly elevated levels of HIF-α and loss of egg-laying function.[7] In *Drosophila*, its single PHD, termed dHPH,[6] hydroxylates Similar (HIF-1α homolog) and causes its oxygen-dependent degradation.[57,58] *Drosophila* PHD is essential for cell growth in fat bodies and wing imaginal discs.[58,59] In humans, heterozygous P317R mutation in PHD2 is associated with moderate polycythemia.[60] P317 is only two amino-acid residues downstream from D315, which directly coordinates Fe(II),[7,10] so P317R mutation likely reduces hydroxylase activity by altering Fe(II) and substrate binding. In addition, R371H mutation in human PHD2 is associated with erythrocytosis, due to reduced binding to, and therefore hydroxylation of, HIF-1α.[61]

PHDs are also implicated to function as tumor suppressors. For example, ectopic overexpression of PHD1 suppresses HIF-1α accumulation and tumor growth,[62] and immortality of human endometrial cancer cells is often associated with loss of PHD2, while re-introduction of PHD2 induced senescence.[63] Likewise, PHD3 is also a potential tumor suppressor. During normal development, many precursors of sympathetic neurons undergo apoptosis, for which PHD3 is uniquely essential.[64] Loss of PHD3 is associated with failure of apoptosis and development of pheochromocytomas.[64] Interestingly, over-expression of PHD3 (but not PHD1 and PHD2) in PC12 cells promoted apoptosis upon NGF withdrawal.[64] Although PHD3 hydroxylase activity is required for this function, expression of mutant HIF-α subunits that cannot be hydroxylated did not prevent PHD3-mediated apoptosis, suggesting that a novel hydroxylase substrate may be involved.

Besides PHD3, PHD2 may also promote cell death, but in a different cell type. Following ischemia–reperfusion treatment of murine heart explants, large infarcts are typically formed due to extensive death of cardiac cells. PHD2 knockdown significantly reduced infarct sizes in ischemia–reperfusion-treated hearts, and such a protective effect may be mediated by HIF-1α stabilization and HIF-mediated expression of iNOS.[65] However, PHDs may have apparently opposite effects in different cell types. In maturing chondrocytes, for example, PHDs promote survival rather than apoptosis. Dimethyloxalylglycine (DMOG), which inhibits all three PHDs, sensitized chondrocytes to H_2O_2-induced apoptosis, whereas HIF-1α knock down by siRNA conferred resistance to H_2O_2.[66]

PHDs may also regulate cell differentiation. For instance, C2C12 skeletal myoblast differentiation is accompanied by increased expression of PHD3, and differentiation is blocked by siRNA-mediated knock down of PHD3.[67] Interestingly, PHD3 directly binds to myogenin and protects it from VHL-dependent polyubiquitination. Thus, HIF-α hydroxylation is not directly involved in this process, although it remains to be determined if PHD3 directly hydroxylates myogenin. In another example, PHDs were shown to be important for the maintenance of osteoblast identity, because inhibition of PHD activities with DMOG caused osteoblasts to adopt adipogenic phenotypes.[68]

We have recently investigated roles of PHDs in mice. While *Phd1*$^{-/-}$ or *Phd3*$^{-/-}$ mice survive normally, *Phd2*$^{-/-}$ embryos die at mid-gestation stages.[69] Major defects include under-developed myocardium and trabeculae in the heart, and poor labyrinthine branching in the placenta, but embryonic vascu-larization was apparently normal. Interestingly, significantly increased HIF-1α, HIF-2α and VEGF-A levels were detected in the embryo proper but not specifically in the heart, suggesting either the involvement of a systemic effect of PHD2 deficiency or a HIF-independent role in heart develop-ment. In adults, tamoxifen-induced, Cre-*loxP*-mediated *Phd2* knockout in a broad range of tissues led to increased vascular density and lumen sizes, accompanied by efficient recruit-ment of vascular smooth muscle cells.[39]

Surprisingly, even though *Phd2* knockout essentially failed in the brain, likely due to blood–brain barrier to tamoxifen, vascular overgrowth occurred similarly as in other tissues.[39] Furthermore, increased VEGF-A was found in the serum but not in local tissues where vascular overgrowth was present,

and other important angiogenic molecules such as angio-poietin-1 and VEGF receptor-2, were not significantly increased. These findings may be explained by proposing systemic effect where circulating angiogenic factors such as VEGF-A and erythropoietin (EPO, which is also increased) might have potential contribution to increased vascular growth.[39]

PHD2-deficient adult mice overexpressed EPO by ~230-fold and developed severe polycythemia.[40] Interestingly, besides an expansion of Ter119$^+$ erythropoietic progenitor cell population, liver- and spleen-derived Lin$^-$ Sca-1$^+$ CD117$^+$ cell population (enriched in hematopoietic stem cells) was also greatly expanded, which had increased hematopoietic activity *in vitro*. Thus, PHD2 deficiency might modify microenvironments in the liver and spleen and activate extramedullary hematopoiesis. A cautionary note, however, is that due to the chronic nature of PHD2 deficiency, it remains to be defined if increased extramedullary hematopoiesis is a primary or secondary defect. Adult *Phd1*$^{-/-}$/*Phd3*$^{-/-}$ double knockout mice also displayed polycythemia, although milder than in PHD2-deficient mice.[40] Surprisingly, renal and serum EPO levels decreased significantly; however, hepatic EPO expression and erythropoietic progenitor numbers did increase. In contrast to PHD2-deficient adult mice where only increased HIF-1α was detected in nuclear extracts, *Phd1*$^{-/-}$/*Phd3*$^{-/-}$ double knockout mice displayed increased HIF-2α but not HIF-1α levels in the liver, which is consistent with cell culture studies, indicating that PHD1 and PHD3 were more efficient on HIF-2α.[34]

Concluding Remarks

The major driving force behind the intensive interest in prolyl hydroxylases is their potential to serve as therapeutic targets to treat various diseases. One prospective application is to promote angiogenesis in ischemic tissues, for which proof of principle has been demonstrated in mice by inhibiting HIF-α hydroxylation using small molecule hydroxylase inhibitors[70] or competitive peptides containing HIF-ODD sequences.[71] Other therapeutic applications are also promising. For example, a recent study reported that TM6008, a novel inhibitor that binds to PHD active site and chelates Fe(II), reduced neuronal cell death in a model of cerebrovascular disease without affecting angiogenesis.[72] Also, FG-2216, an orally available PHD inhibitor, effectively and reversibly promotes erythropoiesis in rhesus macaques by inducing EPO expression.[73] FG-2216 is now in clinical development to treat anemia (http://www.fibrogen.com/trials/). While these findings are all encouraging, it should be cautioned that PHDs are also potential tumor suppressors and it remains to be determined if long-term exposure to PHD inhibitors may favor tumor development. Nonetheless, given the presence of three PHDs, and now the discovery of a fourth related hydroxylase, P4H-TM,[11] it may be possible to exploit differential tissue expression profiles and HIF-α specificity to achieve the best therapeutic outcomes while minimizing associated side effects.

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