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Original Article

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PHD3 inhibits cell proliferation through hydroxylation of PAX2 at proline 9

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Abstract

The oncoprotein transcription factor paired box 2 (PAX2) is aberrantly expressed in cancers, but the underlying mechanism remains elusive. Prolyl hydroxylase 3 (PHD3) hydroxylates the proline residue of HIFα, mediating HIFα degradation. The von Hippel-Lindau protein (pVHL) is an E3 ligase which mediates ubiquitination and degradation of hydroxylated HIFα. PHD3 and pVHL are found to inhibit the expression of PAX2, however, the molecular mechanism is unclear. Here we demonstrate that PHD3 hydroxylates PAX2 at proline 9, which is required for pVHL to mediate PAX2 ubiquitination and degradation. Overexpression of PHD3 enhances prolyl hydroxylation, ubiquitination and degradation of PAX2 with little effect on those of PAX2(P9A). PHD3 does not influence PAX2 expression in *VHL*-null cells. pVHL binds to PAX2 and enhances PAX2 ubiquitination and degradation. However, pVHL does not bind with PAX2(P9A) and cannot enhance its ubiquitination and degradation. Our results suggest that proline 9 hydroxylation is a prerequisite for PAX2 degradation by pVHL. Functional studies indicate that introduction of PAX2 into *PAX2*-null COS-7 cells promotes cell proliferation, which is suppressed by co-expression of PHD3 but not by hydroxylase-deficient PHD3(H196A). PHD3 inhibits PAX2-induced, but not PAX2(P9A)-induced proliferation of COS-7 cells. These results suggest that PHD3 hydroxylates PAX2, followed by pVHL-mediated PAX2 ubiquitination and degradation. This study also suggests that PHD3 inhibits cell proliferation through downregulating PAX2.

Key words PHD3, PAX2, pVHL, prolyl hydroxylation, cell proliferation

Introduction

The prolyl hydroxylases (PHDs) are dioxygenases that use oxygen and 2-oxoglutarate (2-OG) as co-substrates [1,2]. PHDs are involved in the cellular response to oxygen by hydroxylating conserved prolyl residues of hypoxia inducible factor α (HIFα) [3–5]. The hydroxylated HIFα is recognized by the von Hippel-Lindau protein (pVHL) and then subjected to ubiquitin-dependent degradation [6,7]. Under hypoxia, the enzymatic activity of PHDs is inhibited, leading to accumulation of HIFα. HIFα dimerizes with HIFβ and translocates into the nucleus to activate the transcription of genes [8]. These properties make PHDs well suited to act as oxygen sensors.

A few studies indicate that PHD3 has substrates other than HIFα. PHD3 mediates oxygen-dependent stability of activating transcriptional factor 4 (ATF4) [9] and β_2-adrenergic receptor (β_2-AR) [10], and prevents the degradation of myogenin [11]. PHD3 regulates DNA damage response through hydroxylating the human homolog of the *Caenorhabditis elegans* biological clock protein CLK-2, leading to subsequent activation of ATR/CHK1/p53 [12]. PKM2 [13], Spry2 [14], actin [15], ceramide kinase like protein (CERKL) [16], thyroid hormone receptor α (TRα) [17], erythropoietin receptor (EPOR) [18], MAPK6 [19], p53 [20], FOXO3 [21], and acetyl-CoA carboxylase 2 (ACC2) [22] were also found to be the targets of PHD3.

Paired box (PAX) genes encode a set of transcription factors. Nine members of the family have been identified in mammals [23,24]. They are regulators of tissue development and cellular differentiation in embryos, acting to promote cell proliferation, migration and survival [23,24]. Generally, the expression of *PAX* genes is attenuated when tissue development is complete. The PAX gene family is classified into subgroups (I-IV) based on their structures. PAX2, a member of subgroup II of the PAX gene family, is a regulator of

embryogenesis. It may exhibit oncogenic properties when aberrantly expressed in adult tissues. The abnormal re-expression of PAX2 is observed in a variety of cancers including leukemia, breast, prostate, kidney, and bladder carcinoma [23–26]. Heterozygous *PAX2*$^{+/-}$ mutations cause renal hypoplasia and increase apoptosis of the ureteric bud, suggesting an anti-apoptotic function of PAX2 during nephrogenesis [27]. The anti-apoptotic function of PAX2 contributes to the resistance of renal carcinoma cells to chemotherapy [28,29]. Inhibition of PAX2 expression triggers growth inhibition and cell death in renal cell carcinoma cells [30], ovarian cancer and bladder cancer cells [25]. Similarly, inhibition of PAX2 in Kaposi sarcoma cells leads to cell death and reduces cell motility and invasiveness when challenged by serum deprivation or vincristine treatment [31]. PAX2 has become a hallmark of malignant cells, suggesting that it might be a potential target for cancer therapy.

Although elevated expression of PAX2 has been observed in cancer cells, the underlying mechanism is unclear. Luu *et al.* [26] showed that loss of VHL and hypoxia provoked the production of PAX2 at a transcriptional level, probably through HIFα. We found that PHD3 inhibited the expression of PAX2 in the presence of oxygen [32]. These results suggest that PHD3 and pVHL are involved in regulation of PAX2 expression. However, the underlying molecular mechanism is unclear. Here we demonstrate that PHD3 hydroxylates PAX2 at residue proline 9. The hydroxylated PAX2 is recognized by pVHL and subject to ubiquitin-dependent degradation. The PHD3-mediated hydroxylation and the subsequent degradation of PAX2 inhibit the proliferation of cells. These results uncover the molecular mechanism underlying the regulation of PAX2 expression by PHD3 and pVHL.

Materials and Methods

Cell culture and reagents

Human colon cancer HCT116, human embryonic kidney 293T cells and African green monkey SV40-transformed kidney fibroblast COS-7 (PAX2-null) cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM medium (Gibco, Carlsbad, USA) supplemented with 10% serum, 100 U/mL penicillin, and 100 Ug/mL streptomycin. The renal cancer 786-O cells (Type Culture Collection of the Chinese Academy of Sciences) were grown in RPMI 1640 medium (Gibco) supplemented with 10% serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37°C with 5% CO_2. Dimethyloxaloylglycine (DMOG) was purchased from Frontier Scientific (Logan, USA).

Construction of plasmids

The plasmid encoding PAX2 was constructed as previously described [32]. The plasmid encoding PAX2(P9A) was generated by site-directed mutagenesis. The plasmids encoding wild-type PHD3 and hydroxylase-deficient PHD3(H196A), PHD3(H135A) and PHD3 (H135A/H196A) were generated as previously described [33]. The plasmid encoding pVHL was obtained through PCR and cloned into vector pCMVtag2B (Youbio Biological Technology, Changsha, China). Glutathione Stransferase (GST)-VHL fusion proteins were constructed by inserting PCR-generated DNA fragments encoding pVHL into pGEX4T1 vector (Youbio Biological Technology). To produce GST-VHL proteins, the *E. coli* BL21-Gold(DE3)pLysS cells were transformed with pGEX4T1 expression vectors and incubated in the presence of 0.1 mM isopropyl-D-thiogalactoside for 4 h.

Western blot analysis

Total proteins were extracted with RIPA lysis buffer containing protease inhibitors. The protein concentration was determined using BCA reagent kit (Invitrogen, Carlsbad, USA). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA). Membranes were blocked with 5% skim milk, and then incubated with primary antibody at 4°C overnight. Membranes were then washed with Tris-buffered saline containing Tween 20, incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz, Santa Cruz, USA) for 1 h at room temperature, and developed with enhanced chemiluminescence kit (Microwell, Billerica, USA). β-Actin served as the loading control. Antibody against pVHL was from BD Biosciences (San Jose, USA). Anti-PHD3 antibody was from Novus Biologicals (Littleton, USA). The pan-hydroxylated proline antibody was obtained from Advanced Targeting Systems (San Diego, USA). Antibodies against PAX2, HA and Myc were products of Santa Cruz (Santa Cruz, USA). Anti-β-actin and anti-Flag antibodies were from Sigma (St Louis, USA) and Abmart (Shanghai, China), respectively.

Immunoprecipitation assay

The cells were collected and centrifuged at 500 *g* for 5 min at 4°C. The cell pellets were resuspended in ice-cold cell lysis buffer and incubated on ice for 10 min, followed by centrifugation at 13,000 *g* for 15 min. The supernatant was transferred into a new tube, and divided into two parts. One part was used as input to detect the expression of total protein in cells without antibody enrichment. The remainder was incubated with antibody (1 μg of antibody per 500 μg of cell lysate) and Protein A/G-Sepharose beads (40 μl; Santa Cruz) at 4°C overnight on a shake to mix well. The beads were washed 4 times with pre-cooled cell lysis buffer and boiled with 40 μl of 2 × SDS loading buffer for 5 min to obtain denatured proteins. Finally, the obtained samples were subject to western blot analysis.

Real-time PCR

Real-time PCR was performed using standard protocols. In brief, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). RNA concentration was determined with a Nonodrop spectrophotometer, and each paired samples were adjusted to the same concentration. For quantitative PCR (qPCR), cDNA was synthesized using a One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). qPCR was performed using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) on an ABI Stepone Plus system (ABI, Foster City, USA). The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. *β-actin* was used as the internal control. The primers are as follows: *PAX2*: 5′-CCTCGCTCCAATGGTGAGAA-3′ (F), 5′-TGCTGCTGGGTGAAGGTGTC-3′ (R); *β-actin*: 5′-GATCATTGCTCCTCCTGAGC-3′ (F), 5′-ACTCCTGCTTGCTGATCCAC-3′ (R).

Small interfering RNA and cell transfeciton

The PHD3 siRNA and scrambled control siRNA oligonucleotides were designed as previously described [34] and these siRNA oligonucleotides were synthesized by Gene-Pharma (Shanghai, China). The sense sequences of these oligonucleotides are: siPHD3-1: 5′-GUGAUGGUCGCUGCAUCATT-3′; siPHD3-2: 5′-GGAGAGGUCU

AAGGCAAUGTT-3′; siVHL-1: 5′-GCUCUACGAAGAUCUGGAATT-3′, siVHL-2: 5′-GCCAGUGUAUACUCUGAAAT-3′; siVHL-3: 5′-CUAG UCAAGCCUGAGAAUUTT-3′; Control: 5′-UUCUCCGAACGUGUCA CGUTT-3′. siRNA oligos were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

Liquid chromatography–tandem mass spectrometry

The 293T cells were co-transfected with plasmids encoding Myc-PAX2 and PHD3. Twenty-four hours post-transfection, the cells were harvested. Cleared cell lysates were incubated with anti-Myc antibody at 4°C for 3 h, then the protein A/G PLUS agarose beads (Santa Cruz) were added into the mixture, followed by incubation overnight at 4°C. The beads were washed 4 times with ice-cold PBS, and proteins were eluted by boiling the beads in SDS-containing sample buffer. The protein samples were resolved by SDS-PAGE and identified by Commassie bright blue staining. Protein bands of interest were excised for analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS) which was performed in the Research Center for Protein Analysis, Shanghai Institutes of Materia Medica, Chinese Academy of Sciences (Shanghai, China). A +16-dalton mass shift of a peptide containing proline may indicate prolyl hydroxylation.

Cell proliferation assay

Cell proliferation was determined by CCK8 assay as previously described [35]. Briefly, cells were seeded into 96-well plates at 3×10^3 cells/well and cultured. At 0, 24, 48, 72, and 96 h after seeding, the culture medium was removed and 90 μL fresh complete medium and 10 μL CCK8 reagent (TargetMol, Boston, USA) were added to each well, followed by incubation for 1 h. The absorbance of each well was measured at 450 nm with a VICTOR® Nivo™ microplate reader (PerkinElmer, Waltham, USA).

GST pull-down assay

GST pull-down assay was performed as previously described [34]. Briefly, bacterial cells were lysed with the following buffer: 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% NP40, pH7.5. The supernatants of *E. coli* containing GST-VHL were incubated with glutathione-Sepharose 4B beads (GE Healthcare, Uppsala, Sweden) at 4°C for 2 h. Then the beads were washed and incubated at 4°C with 293T cell lysates that contained Myc-PAX2 proteins. After 2 h, the beads were washed with ice-cold PBS and proteins were eluted by boiling the beads in SDS-containing sample buffer. The protein samples were subject to western blot analysis.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software package. Data are presented as the mean ± SD, except where indicated. Student's *t*-test was used for continuous variables. $P < 0.05$ was considered significant.

Results

PHD3 inhibits the expression of PAX2 post-transcriptionally in a hydroxylase-dependent manner

Previously, we reported that PHD3 inhibited the expression of PAX2 in colon cancer cells [32]. Here, we found that overexpression of PHD3 decreased the protein level of PAX2 and knockdown of PHD3 enhanced the protein level of PAX2 as well (Figure 1A) in 293T cells. Overexpression and knockdown of PHD3 had no influence on the *PAX2* mRNA level (Figure 1B), implying that PHD3 inhibits the expression of PAX2 at the post-transcriptional level. As PHD3 has prolyl hydroxylase-dependent and hydroxylase-independent functions [36], we determined whether or not the regulation of PAX2 expression is PHD3 prolyl hydroxylase-dependent. It was found that overexpression of the prolyl hydroxylase-deficient PHD3(H135A), PHD3(H196A) or PHD3(H135A/H196A) had little effect on protein level of PAX2 (Figure 1C), indicating that the hydroxylase activity is required for PHD3 to inhibit PAX2 expression. These data suggest that PAX2 is a substrate of PHD3, and PHD3 may directly target PAX2 for its degradation.

PHD3 hydroxylates PAX2 at proline 9

As PHD3 inhibits the expression of PAX2 post-transcriptionally in a hydroxylase-dependent manner, we assumed that PAX2 may be a substrate of PHD3. To identify whether PAX2 is a substrate of PHD3, we performed LC-MS/MS. The experimental procedure is shown in Figure 2A. Using this approach, we identified proline 9 of PAX2 as

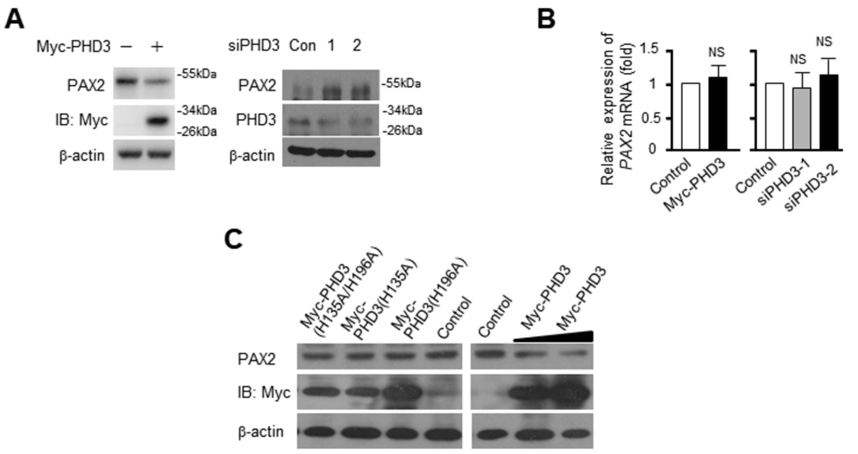

Figure 1. PHD3 inhibits PAX2 expression post-transcriptionally in a hydroxylase-dependent manner (A) Overexpression of PHD3 reduced and knockdown of PHD3 enhanced the protein level of PAX2 in 293T cells. (B) Overexpression or knockdown of PHD3 had no effect on the mRNA level of *PAX2* in 293T cells. (C) In 293T cells, overexpression of PHD3, but not that of PHD3(H135A), PHD3(H196A) or PHD3(H135A/H196A) decreased the protein level of PAX2. NS, no significance.

the hydroxylation residue (Figure 2B). To confirm that PHD3 could hydroxylate proline 9 residue of PAX2, we constructed a plasmid encoding PAX2(P9A) and determined whether PHD3 could influence the hydroxylation of PAX2(P9A) by using a pan-hydroxylated proline antibody. It was found that overexpression of PHD3 enhanced the prolyl hydroxylation level of PAX2, which was inhibited by prolyl hydroxylase inhibitor DMOG (Figure 2C). However, overexpression of PHD3 had little effect on prolyl hydroxylation of PAX2(P9A) (Figure 2D). These results suggest that the proline 9 of PAX2 is the residue that is hydroxylated by PHD3. It was also found that the amino acid sequence flanking proline 9 of PAX2 is evolutionarily conserved (Figure 2E).

PHD3-mediated proline 9 hydroxylation is required for PAX2 degradation

We assumed that hydroxylation of proline 9 is critical for PAX2 degradation. To confirm this, we transfected cells with vectors encoding Myc-PAX2 and PHD3, or with vectors encoding Myc-PAX2 (P9A) and PHD3. As expected, overexpression of PHD3 inhibited the expression of Myc-PAX2, while overexpression of PHD3 had little effect on that of PAX2(P9A) (Figure 3A). These data suggest that proline 9 is required for PHD3-mediated degradation of PAX2. It is known that PHD3 prolyl hydroxylase activity is controlled by oxygen availability and PHD3 loses the activity in the absence of oxygen [3–5]. We next determined the effect of hypoxia on expression of PAX2 and PAX2(P9A). Cells transfected with vector encoding Myc-PAX2 or Myc-PAX2(P9A) were incubated in hypoxic condition. The results showed that hypoxia induced the expression of Myc-PAX2 (Figure 3B). However, hypoxia did not induce that of Myc-PAX2(P9A). These results imply that proline 9 is critical for hypoxia to induce PAX2. We also determined whether proline 9 is critical for PAX2 ubiquitination. It was found that ectopic expression of PHD3 enhanced the ubiquitination of PAX2 (Figure 3C). However, ectopic expression of PHD3 had little effect on that of PAX2(P9A). Thus, proline 9 is critical for PAX2 ubiquitination promoted by PHD3. Together, these results suggest that PHD3-mediated proline 9 hydroxylation is required for PAX2 degradation.

pVHL is involved in PHD3-mediated degradation of PAX2

Both PHD3 and pVHL reduce the protein level of PAX2 [32]. We asked whether pVHL is involved in PHD3-mediated degradation of PAX2. We examined this in 293T and *VHL*-null renal carcinoma 786-O cells. As shown in Figure 4A, 786-O cells did not express pVHL, while 293T cells did. Ectopic expression of pVHL decreased the protein level of PAX2 in both 786-O and 293T cells (Figure 4A). Ectopic expression of PHD3 inhibited the expression of PAX2 only in 293T cells (Figure 4B). However, ectopic expression of PHD3 had little effect on PAX2 expression in *VHL*-null 786-O cells. Knockdown of *VHL* increased the level of PAX2 (Figure 4C), and overexpression of PHD3 did not influence the expression of pVHL (Figure 4D). These results suggest that pVHL is involved in PHD3-mediated degradation of PAX2.

Proline 9 hydroxylation is required for PAX2 ubiquitination and degradation by pVHL

We then explored whether pVHL also has an inhibitory effect on the expression of PAX2(P9A). 293T cells were transfected with vectors encoding pVHL plus PAX2 or pVHL plus PAX(P9A). The results showed that overexpression of pVHL inhibited the expression of PAX2 (Figure 4E). However, ectopic expression of pVHL had little effect on the protein level of PAX2(P9A).

In the regulation of HIF1α expression, hydroxylation of HIF1α is a

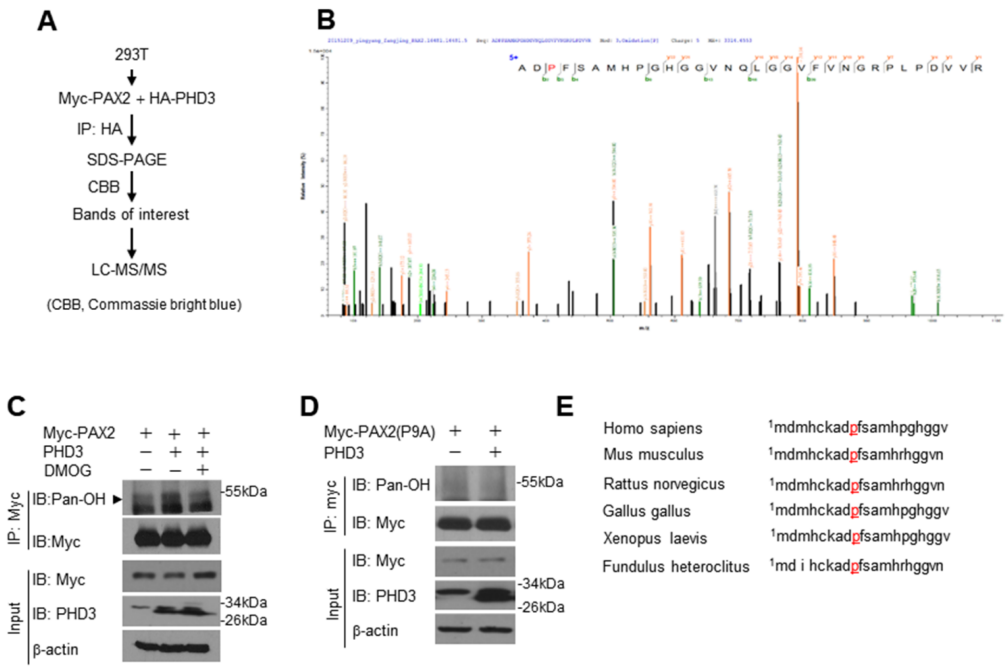

Figure 2. PHD3 hydroxylates PAX2 at proline 9 (A) Schematic of LC-MS/MS analysis of PAX2. (B) LC-MS/MS analysis of prolyl hydroxylation of PAX2. The residue proline 9 in PAX2 was identified as the hydroxylation site. (C) Overexpression of PHD3 increased PAX2 proline hydroxylation, which was inhibited by DMOG. The 293T cells expressing PAX2-myc or PAX2-myc plus PHD3 vectors were treated with DMOG (1 mM) for 4 h, followed by immunoprecipitation with anti-Myc antibody and western blot analysis using pan-hydroxylated proline antibody. (D) Overexpression of PHD3 had little effect on prolyl hydroxylation of PAX2(P9A)-myc in 293T cells. (E) The amino acid sequence flanking proline 9 of PAX2 is evolutionarily conserved.

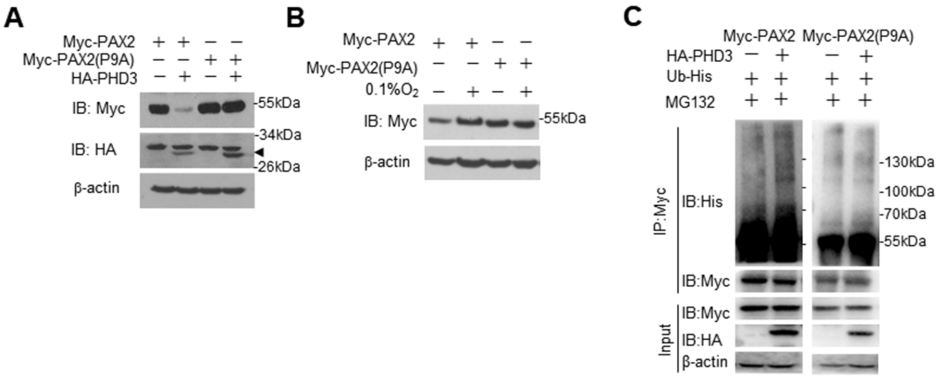

Figure 3. PHD3-mediated proline 9 hydroxylation is required for PAX2 degradation (A) PHD3 did not inhibit the expression of PAX2(P9A) in 293T cells. (B) Hypoxic condition (0.1% O_2) induced the expression of PAX2-myc, but not that of PAX2(P9A)-myc in 293T cells. (C) Overexpression of PHD3 enhanced the level of ubiquitinated PAX2-myc with little effect on that of ubiquitinated of PAX2(P9A)-myc. The 293T cells were transfected as indicated and the cells were incubated with MG132 (10 μM) for 8 h. Ubiquitination of PAX2-myc and PAX2(P9A)-myc was determined by immunoprecipitation and western blot analysis.

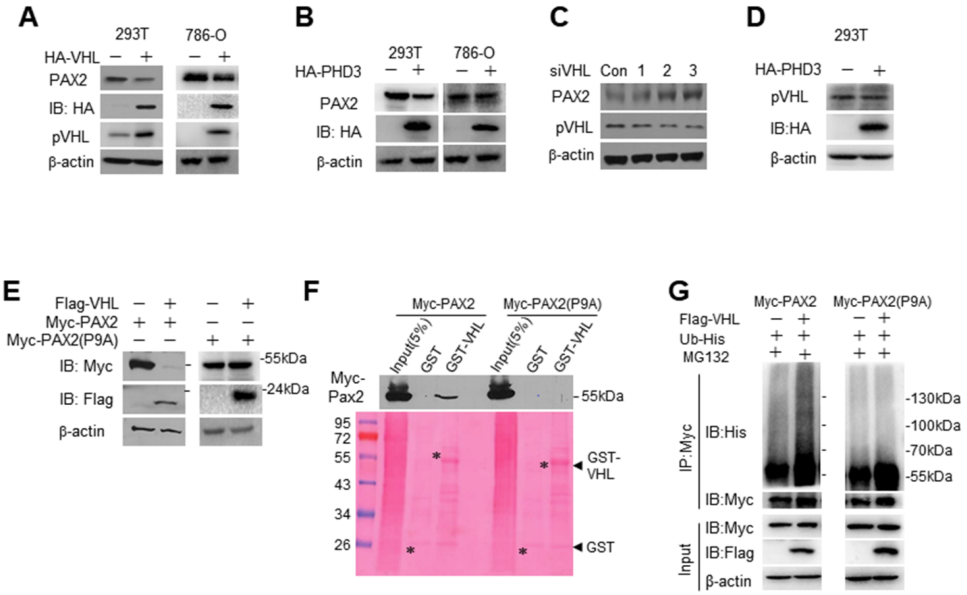

Figure 4. Proline 9 hydroxylation is required for PAX2 degradation by pVHL (A) Overexpression of HA-VHL decreased the protein level of PAX2 in both 786-O and 293T cells. (B) Overexpression of HA-PHD3 inhibited the expression of PAX2 in 293T cells, but not in *VHL*-null 786-O cells. (C) Knockdown of *VHL* with different siVHL oligos increased the protein level of PAX2 in 293T cells. (D) Overexpression of PHD3 did not influence the expression of pVHL. (E) Overexpression of pVHL promoted the degradation of PAX2-myc with little effect on that of PAX2(P9A)-myc in 293T cells. (F) PAX2-myc interacted with GST-VHL but PAX2(P9A)-myc could not interact with GST-VHL. GST pulldown assay was performed as described in Methods. (G) Overexpression of Flag-VHL enhanced the ubiquitination of PAX2-myc with little effect on that of PAX2(P9A)-myc.

prerequisite for pVHL to bind with HIF1α, which mediates the ubiquitination of HIF1α and its subsequent degradation. We previously found that pVHL is associated with PAX2 [32]. Therefore, we determined whether PAX2 hydroxylation at proline 9 is required for the binding of PAX2 to pVHL. The GST-pulldown assay results indicated that PAX2, but not PAX2(P9A), is associated with pVHL (Figure 4F), implying that hydroxylation of proline 9 of PAX2 is a prerequisite for pVHL binding.

Furthermore, we determined whether or not pVHL mediates the ubiquitination of PAX2(P9A). It was found that overexpression of pVHL enhanced the ubiquitination of PAX2 (Figure 4G). However, overexpression of pVHL had little effect on that of PAX2(P9A).

Together, these results suggest that proline 9 hydroxylation of PAX2 is required for pVHL-PAX2 interaction, PAX2 ubiquitination and the subsequent degradation of PAX2.

PHD3 inhibits cell proliferation through downregulating PAX2

PAX2 promotes the proliferation of cancer cells [25,30], while PHD3 represses the proliferation of cancer cells [34]. To evaluate whether PHD3 inhibits cell proliferation through PAX2, we examined African green monkey SV40-transformed kidney fibroblast COS-7 cells. The COS-7 cells are *PAX2*-null and ectopic expression of PAX2 enhanced the proliferation of the cells [37]. The cells were transfected with equal amount of Myc-PAX2 or Myc-PAX2(P9A) plasmid. The results showed that ectopic expression of either PAX2 or PAX2(P9A) pro-

moted the proliferation of COS-7 cells (Figure 5A). Co-expression of PHD3 inhibited the exogenous PAX2-induced COS-7 cell proliferation dramatically (Figure 5A). However, co-expression of PHD3 had little inhibitory effect on COS-7 cell proliferation promoted by PAX2 (P9A) expression. In the presence of HA-PHD3, overexpression of PAX2(P9A) still enhanced cell proliferation significantly. However, in the presence of HA-PHD3, overexpression of PAX2 did not induce significant COS-7 cell proliferation (Figure 5A). Overexpression of PHD3 decreased the level of Myc-PAX2, but it had little effect on the level of PAX2(P9A) (Figure 5B). The protein level of Myc-PAX2 (P9A) is much higher than that of Myc-PAX2. This is probably due to the resistance of PAX2 mutant to PHD3. When the activity of PHD3 was inhibited by DMOG, the level of Myc-PAX2 was identical to that of PAX2(P9A) (Figure 5C), indicating that the original expression of wild-type and mutated PAX2 is the same.

We next determined the effect of ectopic expression of PHD3 and PHD3(H196A) on the proliferation of the COS-7 cells expressing exogenous PAX2. The results showed that overexpression of PHD3 inhibited the proliferation of COS-7 cells promoted by PAX2 (Figure 5D). However, ectopic expression of PHD3(H196A) had little inhibitory effect on COS-7 cell proliferation induced by PAX2. The protein levels of PAX2 in control, PHD3-myc expressing and PHD3 (H196A)-myc expressing COS-7 cells are shown in Figure 5E. The results show that the expression of exogenous PHD3 inhibited PAX2 expression dramatically, while the expression of hydroxylase-deficient PHD3(H196A) had little effect on PAX2 expression, which is consistent with the results of Figure 5D.

Together, these results suggest that PHD3 inhibits cell proliferation through downregulating PAX2.

Discussion

In this study, we demonstrate that PHD3 hydroxylates PAX2 at proline 9, and hydroxylation of proline 9 in PAX2 is required for the tumor suppressor pVHL to mediate the ubiquitination and degradation of PAX2. Our results uncover the molecular mechanism underlying the regulation of PAX2 expression modulated by PHD3 and pVHL.

Aberrant expression of PAX2 has been observed in a variety of cancers. PAX2 exhibits oncogenic properties when aberrantly re-expressed in adult tissues. Luu *et al.* [26] reported that hypoxia provoked PAX2 up-regulation at the transcriptional level probably through HIFα. Using luciferase reporter assay, however, the authors did not obtain any luciferase activity induction in spite of hypoxia responsive element motif in the promoter of *PAX2*. The authors proposed that, in addition to HIFα, other tissue-specific factors might be required for PAX2 induction. We showed previously that PHD3 inhibits PAX2 expression at the post-transcriptional level and pVHL is involved [32]. However, the underlying mechanism is unknown. Considering the fact that the prolyl hydroxylase activity is required for PHD3 to inhibit the expression of PAX2 (Figure 1), we presumed that PHD3 might directly hydroxylate PAX2 for its degradation. The LC-MS/MS results showed that the proline 9 residue of PAX2 is hydroxylated and PHD3 is required for prolyl hydroxylation of PAX2 (Figure 2). Proline 9 is critical for hypoxia to induce PAX2 expression and for PHD3 to promote PAX2 degradation (Figure 3). Our results suggest that hydroxylation of proline 9 is a

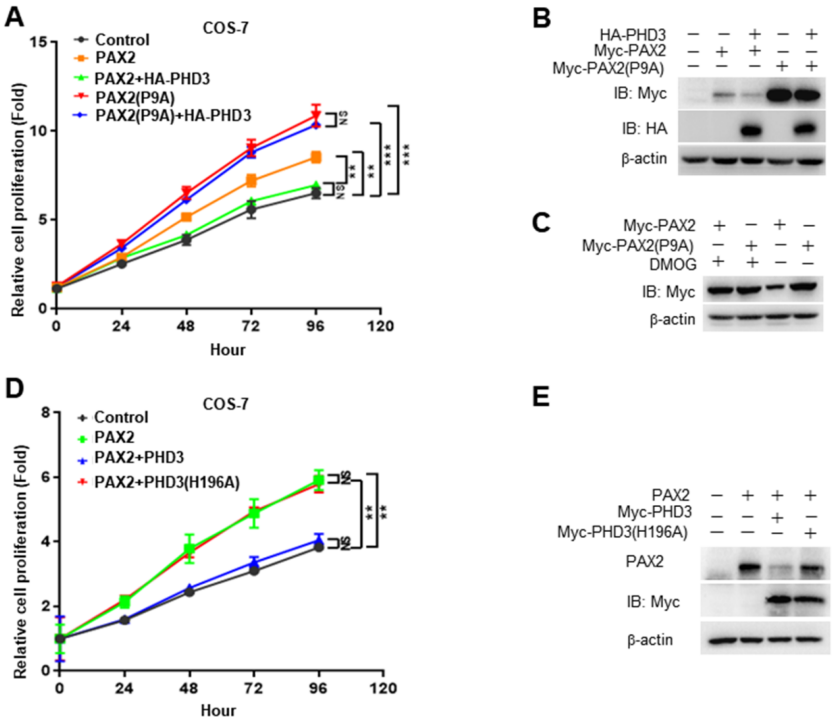

Figure 5. PHD3 inhibits cell proliferation through downregulating PAX2 (A) Ectopic expression of HA-PHD3 inhibits PAX2 overexpression-induced, but not PAX2(P9A) overexpression-induced, proliferation of COS-7 cells. (B) Overexpression of PHD3 decreased protein level of PAX2-myc with little effect on that of PAX2(P9A)-myc in COS-7 cells transfected with vectors encoding PAX2-myc or PAX2(P9A)-myc. (C) Transfection efficiency of PAX2-myc and PAX2(P9A)-myc in COS-7 cells. The cells transfected as in (A) were treated with DMOG (1 mM) for 10 h. The cells were harvested for western blot analysis of PAX2-myc and PAX2(P9A)-myc. (D) Ectopic expression of PHD3, but not PHD3(H196A), inhibited PAX2 overexpression-enhanced proliferation of COS-7 cells. (E) Overexpression of PHD3-myc, but not PHD3(H196A), inhibited the expression of PAX2 in COS-7 cells transfected as indicated. $^{**}P<0.01$, $^{***}P<0.001$. NS, no significance.

prerequisite for PAX2 degradation by PHD3.

It is known that pVHL functions as an E3-ligase and recognizes the PHD-hydroxylated HIFα, leading to HIFα degradation [5,38]. Our previous study showed that pVHL interacts with PAX2 [32]. We therefore presumed that hydroxylation of proline 9 in PAX2 was required for pVHL to mediate PAX2 degradation. As expected, pVHL bound to PAX2 but not PAX2(P9A), and overexpression of pVHL promoted the ubiquitination and degradation of PAX2 with little effect on those of PAX2(P9A) (Figure 4).

Finally, we determined the biological consequence of PHD3-dependent hydroxylation and degradation of PAX2. We employed *PAX2*-null COS-7 cells in the experiment. We found that ectopic expression of either PAX2 or PAX2(P9A) promoted the proliferation of COS-7 cells, and PAX2(P9A) promoted much more than PAX2 did (Figure 5A). This might be due to the fact that PAX2(P9A) is resistant to degradation by endogenous PHD3 and thus the level of PAX2(P9A) is higher than that of PAX2 in the cells. Overexpression of PHD3 dramatically inhibited the PAX2-induced cell proliferation. However, PHD3 had little effect on PAX2(P9A)-induced cell proliferation (Figure 5D). Moreover, we found that ectopic expression of PHD3(H196A) did not inhibit PAX2-promoted COS-7 cell proliferation as PHD3 did (Figure 5D). These results suggest that PHD3 inhibits cell proliferation through hydroxylating and down-regulating PAX2.

PHDs regulate cellular responses to hypoxia and are recognized as oxygen sensors. They mediate a wide variety of cellular events by hydroxylating a growing list of protein substrates. In this study, we demonstrated that PHD3 hydroxylates PAX2 at proline 9, which leads to PAX2 ubiquitination and degradation by pVHL. Our findings provide insight into the regulation of PAX2 expression by PHD3 and expand our understanding of the function of prolyl hydroxylation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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