Original Article

http://www.jcpjournal.org
pISSN 2288-3649 · eISSN 2288-3657
https://doi.org/10.15430/JCP.24.031

JCP

Non-canonical Function of Prolyl Hydroxylase Domain 2 in Breast Cancer Cell Growth and Progression: Role of Peptidyl-prolyl Cis-trans Isomerase NIMA-interacting 1

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Prolyl hydroxylase domain 2 (PHD2) is the primary oxygen sensing enzyme involved in hydroxylation of hypoxia-inducible factor (HIF). Under normoxic conditions, PHD2 hydroxylates specific proline residues in HIF-1α and HIF-2α, promoting their ubiquitination and subsequent proteasomal degradation. Although PHD2 activity decreases in hypoxia, notable residual activity persists, but its function in these conditions remains unclear. Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) targets proteins with phosphorylated serine/threonine-proline (pSer/Thr-Pro) motifs. As PHD2 contains several pSer/Thr-Pro motifs, it may be a potential substrate of Pin1. In the present study, we found Pin1 and PHD2 interactions in human breast cancer MDA-MB-231 cells. The breast cancer tissue array revealed higher levels of PHD2 and Pin1 in tumors compared to adjacent normal tissues. Through liquid chromatography-tandem mass spectrometry spectrometry, three phosphorylation sites (S125, T168, and S174) on PHD2 were identified, with serine 125 as the main site for Pin1 binding. As a new Pin1 binding partner, oncogenic PHD2 could be a potential therapeutic target for breast cancer treatment.

Key Words Breast cancer, Hypoxia-inducible factor, Prolyl hydroxylase domain 2, Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1, Protein-protein interaction

INTRODUCTION

Prolyl hydroxylase domains (PHDs) catalyzes the hydroxylation of hypoxia-inducible factors (HIFs) at particular proline residues using molecular oxygen, leading to their degradation through the ubiquitin-proteasome pathway in normoxic conditions (normoxia) [1-3]. PHD2, one of the three PHD isoforms, is the primary physiological oxygen sensor responsible for hydroxylation of HIF proteins [1,4]. Regulation of oxygen homeostasis is critical in normal physiology; however, tissue oxygen tension is compromised in some pathologic conditions including cancer [5].

The differential regulation of PHD2 activity in tumor progression, tumor vasculature and metastasis versus in normal physiology has been explored [6-8]. Under hypoxia, PHD2 activity is reduced, leading to increased HIF levels, although residual PHD2 activity is still present [9]. Notably, PHD2 haplodeficiency has been shown to reduce metastasis in cancer models [7]. In addition to its canonical function as an oxygen sensor, PHD2 also engages in various signaling pathways, independently of its hydroxylase function [10,11], such as binding directly with epidermal growth factor receptor (EGFR) in breast cancer cells, thus stabilizing EGFR and maintaining its activity [12].

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is a unique *cis-trans* isomerase that binds specifically

Received December 9, 2024, Revised December 22, 2024, Accepted December 23, 2024, Published on December 30, 2024
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to phospho-serine/threonine-proline (pSer/Thr-Pro) sequences modifying substrate protein conformation, stability and function [13,14]. Originally identified as a cell cycle protein, Pin1 is often overexpressed in the tumors [15,16], where it promotes cancer progression by modulating multiple pro-oncogenic and tumor suppressive pathways, leading to poor clinical outcomes [17-19]. Pin1 regulates key oncoproteins including rat sarcoma virus, neurogenic locus notch homolog proteins, early region 2 binding factor, phosphatidylinositol-3-kinase (PI3K), etc., and regulates pathways governing hallmarks of cancer [20-23]. Therefore, Pin1 is a promising therapeutic target [24-27].

Both PHD2 and Pin1 are pivotal in HIF regulation. PHD2 hampers HIF accumulation under normoxia while Pin1 binds and stabilizes HIF-1α in a phosphorylation-dependent way [28]. We have previously shown that Pin1 inhibition reduces tumor growth, angiogenesis and hypoxia-induced HIF-1α upregulation [28]. Very recently, we have reported that Pin1 also stabilizes HIF-2α in an oxygen-independent manner in breast cancer cells and tissues [29]. Given the roles of PHD2 and Pin1 in HIF regulation and their overexpression in breast cancer, we aimed to explore the association between PHD2 and Pin1 in the context of breast cancer progression.

MATERIALS AND METHODS

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, FBS, and penicillin/streptomycin were the products of Gibco BRL. Stealth™ RNAi-negative control duplexes and Trizol® were obtained from Invitrogen Life Technologies Corporation. A human breast cancer tissue microarray (LVI5050) was provided by US Biomax. Rabbit polyclonal antibodies against HIF-1α, HIF-2α, PHD2, and PHD3 were sourced from Novus Biologicals, while the PHD1 antibody was purchased from abcam. Antibodies targeting Pin1, actin, lamin B_1, and α-tubulin were provided by Santa Cruz Biotechnology, Inc. The ubiquitin antibody came from Cell Signaling Technology, and the secondary antibodies were purchased from Zymed Laboratories.

Cell culture

Human breast cancer cell lines (MDA-MB-231 and MCF7) and HEK293T cells were obtained from the American Type Culture Collection. MDA-MB-231 and HEK293T cells cultured in DMEM while MCF7 cells maintained in RPMI medium. All cells lines were grown in media containing 5% FBS and 100 ng/mL antibiotics at 37°C in an incubator with 5% CO_2 and 95% air. Hypoxic conditions were achieved by culturing cells in 1% O_2 environment.

Small interfering RNA and DNA plasmid transfection

Pin1 small interfering RNA (siRNA) #1 was sourced from Bioneer, Inc. Control siRNA, Pin1 siRNA #2 (sc-36230), and HIF PHD2 siRNA (sc-45537) were supplied by Santa Cruz Biotechnology, Inc. Lipofectamine RNAiMAX was obtained from Life Technologies Corporation and transfected for 48 hours into breast cancer cells. Full-length and mutants PHD2 were made by Cosmo Genetech Company. HEK293T cells were grown until 90% confluence, and Lipofectamine 2000 obtained by Thermo Fisher Scientific, Inc. was used to transfect these cells with wild type (WT) HA-PHD2 and pcDNA-Pin1 constructs according to the manufacturer's instructions. Western blot and immunoprecipitation assays were conducted 24 hours post-transfection.

Quantitative real-time PCR analysis

Total RNA from MDA-MB-231 cells was extracted using the Trizol® reagent from Invitrogen. RNA quality was assessed with the RNA 600 nano chip from Agilent Technologies, and quantified using the ND-2000 spectrophotometer from Thermo Inc. Gene expression levels of Pin1, PHD1, PHD2, and PHD3 were measured by real-time quantitative PCR (qPCR) using a standardized protocol with the RealHelix™ SYBR Green I qPCR kit from NanoHelix Co., Ltd. Fluorescent signals were detected with the 7500 Fast Real-time PCR system, and expression levels were quantified using the comparative cycle threshold method. The PCR primer sequences for the qPCR were: *Pin1*, 5'-TGA TCA ACG GCT ACA TCC AG-3' (F) and 5'-CAA ACG AGG CGT CTT CAA AT-3' (R); *PHD1*, 5'--GGC AACT ACG TCA TCA ATG GG-3' (F) and 3'-TGG GGA TTG TCA ACA TGC CTC-5' (R); *PHD2,* 5'-TTG TTA CCC AGG CAA CGG AAC-3' (F) and 3'-CCT TGG CGT CCC AGT CTTT-5' (R); *PHD3,* 5'-GGC TGG GCA AAT ACT ATG TCAA-3' (F) and 3'-GGT TGT CCA CAT GGC GAACA-5' (R); *GAPDH*, 5'- CAT GAG AAG TAT GAC AAC AGC CT-3' (F) and 5' -AGT CCT TCC ACG ATA CCA AAG T-3' (R).

Western blot analysis

Cells were lysed and centrifuged at 18,000 x *g* for 20 minutes. Protein concentration was measured using the BCA protein kit (Pierce). Protein (30 μg) was separated by SDS-PAGE. The protein blots were blocked at 37°C for 1 hour with 5% skim dry milk prepared in PBS with 0.1% Tween-20 buffer. Blocked membranes were then incubated overnight at 4°C with primary antibodies against Pin1 and PHD2.

Preparation of nuclear and cytoplasmic extracts

Cells were washed with cold PBS and then suspended in hypotonic buffer A on ice, following a previously reported protocol [29]. After centrifugation, the supernatant containing the cytosolic fractions was collected. The remaining cell pellet was washed twice with buffer A and resuspended in buffer C. This suspension was incubated on ice for 1 hour, followed by centrifugation at 18,000 x *g* for 15 minutes. The nuclear extracts obtained were stored at –70°C until further use.

HEK293T and MDA-MB-231 cells were lysed, and total

proteins (80 μg) were incubated with primary antibodies overnight at 4°C. Protein A/G-PLUS Agarose beads from Santa Cruz Biotechnology were then added for precipitation. After centrifugation at 1,000 x *g* for 1 minute, the supernatant was removed, and the precipitated beads were washed in cell lysis buffer. The immunoprecipitated beads were subsequently prepared according to a previously described protocol [30].

In situ proximity ligation assay

The DuoLink™ kit from Sigma-Aldrich was used to perform the proximity ligation assay (PLA). HEK293T cells were transfected with HA-PHD2/pcDNA-Pin1, control siRNA or Pin1 siRNA for 48 hours. The cells were fixed, permeabilized, blocked with a solution of 0.1% Triton in PBS containing 5% bovine serum albumin and incubated overnight at 4°C with Pin1 monoclonal (1:100) and PHD2 polyclonal (1:200) antibodies. PLA affinity probes (PLUS and MINUS) were subsequently added and incubated at 37°C for 1 hour. The probes were visualized using fluorescence microscopy from Nikon [29,30].

Tissue array analysis

Paraffin-embedded human breast cancer tissue arrays (Cat. No. BC08118a provided by US Biomax, Inc.), including adjacent normal tissues, were deparaffinized with xylene, and rehydrated through a series of ethanol baths (100%, 90%, 80%, and 70%). Antigen retrieval was performed by boiling the tissue sections in hot citrate buffer for 30 minutes, followed by permeabilization and blocking according to a standard protocol. The tissue sections were washed in PBS and incubated overnight at 4°C antibodies against Pin1 and PHD2. Afterward, the sections were treated fluorescently conjugated secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated for PHD2, green signal; tetramethylrhodamine isothiocyanate [TRITC]-conjugated for Pin1, red signal) for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and images were captured using a fluorescent microscope from Nikon.

Immunofluorescence microscopy

For immunocytochemistry staining, MDA-MB-231 cells were plated in an 8-chambered plate at a density of 1 x 10^4 cells per well. Once the cells reached the 80% confluency, they were fixed, permeabilized and blocked before being incubated overnight with anti-Pin1 and anti-PHD2 antibodies. The cells were then incubated with fluorophore-conjugated secondary antibodies (FITC and TRITC). DAPI staining was used to label the nuclei. The slides were scanned, and the stained cells were visualized using a Nikon fluorescent microscope.

Clonogenic assay

For the clonogenic assay, MDA-MB-231 and MCF7 cells were plated in 6-well plates at a density of 150 to 200 cells per well and cultured for 14 days prior to transfection with control siRNA, Pin1 siRNA, or PHD2 siRNA for 48 hours with media changes every other day. Following the 14-day incubation, colonies were fixed with methanol at 4°C for 1 hour and stained with 0.05% crystal violet (Sigma-Aldrich) for additional 4 hours. The excess dye was washed off with PBS, and colonies were visualized and counted using LAS-4000 image reader (Nikon) [29].

Migration assay

To assess the cell migration, MCF7 cells were pretreated with control, Pin1 or PHD2 siRNAs then plated into Culture-Inserts® (ibid). After 24 hours, when the cells had adhered to the inserts, they were gently removed using sterile tweezers. The ability of the cells to migrate was then monitored at various time points under a microscope (Nikon).

Identification of phosphorylation sites on PHD2

To identify potential phosphorylation sites, MDA-MB-231 cells were transfected with HA-PHD2, and immunoprecipitated samples were analyzed by SDS-PAGE. The bands containing HA-PHD2 were excised, and proteins were eluted through trypsin digestion [31]. Phosphorylation of HA-PHD2 was examined in peptides using a hybrid quadrupole linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos). MS/MS spectra were searched against a database of all translated human open reading frames and their reversed sequences using the SEQUEST algorithm. Mass spectrometry data were captured and processed with Proteome Discoverer 2.5 software (Thermo Fisher Scientific Inc.).

Survival analysis

Gene expression data (in tpm) and clinical information for the entire TCGA-BRCA cohort were obtained from the UCSC Xena Browser (https://xenabrowser.net/datapages/). Patients were categorized into high and low PHD2 (Egln1) expression groups based on log2 (tpm + 0.001) values, using the 10% and 90% percentiles as cut-offs. Survival analysis was performed using overall survival time in R with "survival" and "survminer" packages and visualized with ggsurvplots. Additionally, survival analysis based on PHD2 mRNA expression was conducted using METABRIC data, where patients were sub-grouped into PHD2 (Egln1) high and low groups based on the median value of PHD2 mRNA expression level.

Network analysis of protein-protein interaction

Protein-protein interactions were analyzed using the STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org).

Sequence conservation of protein

Protein sequence conservation was analyzed by Multiple Sequence Alignment (MSA) using the MUSCLE tool (Multiple Sequence Comparison by Log- Expectation, https://www.ebi.

ac.uk/Tools/msa/muscle/.

Statistical analysis

All data are presented as the mean ± SD from at least three independent experiments. Statistical analysis was performed using one-way ANOVA or two-tailed unpaired Student's *t*-tests, with significance indicated as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001; 'ns' denotes non-significant results. Analyses were conducted using GraphPad Prism 8.0 software (GraphPad Software).

RESULTS

Overexpression of PHD2 and Pin1 and their functional relationship in breast cancer

Breast cancer tissue microarrays revealed overexpression of both Pin1 and PHD2 in tumor samples (Fig. 1A and 1B), and there is a strong positive correlation between Pin1 and PHD2 expression (Fig. 1C). However, there was no significant correlation of Pin1 or PHD2 expression with patient characteristics like tumor stage or patient age (Figure S1A and S1B).

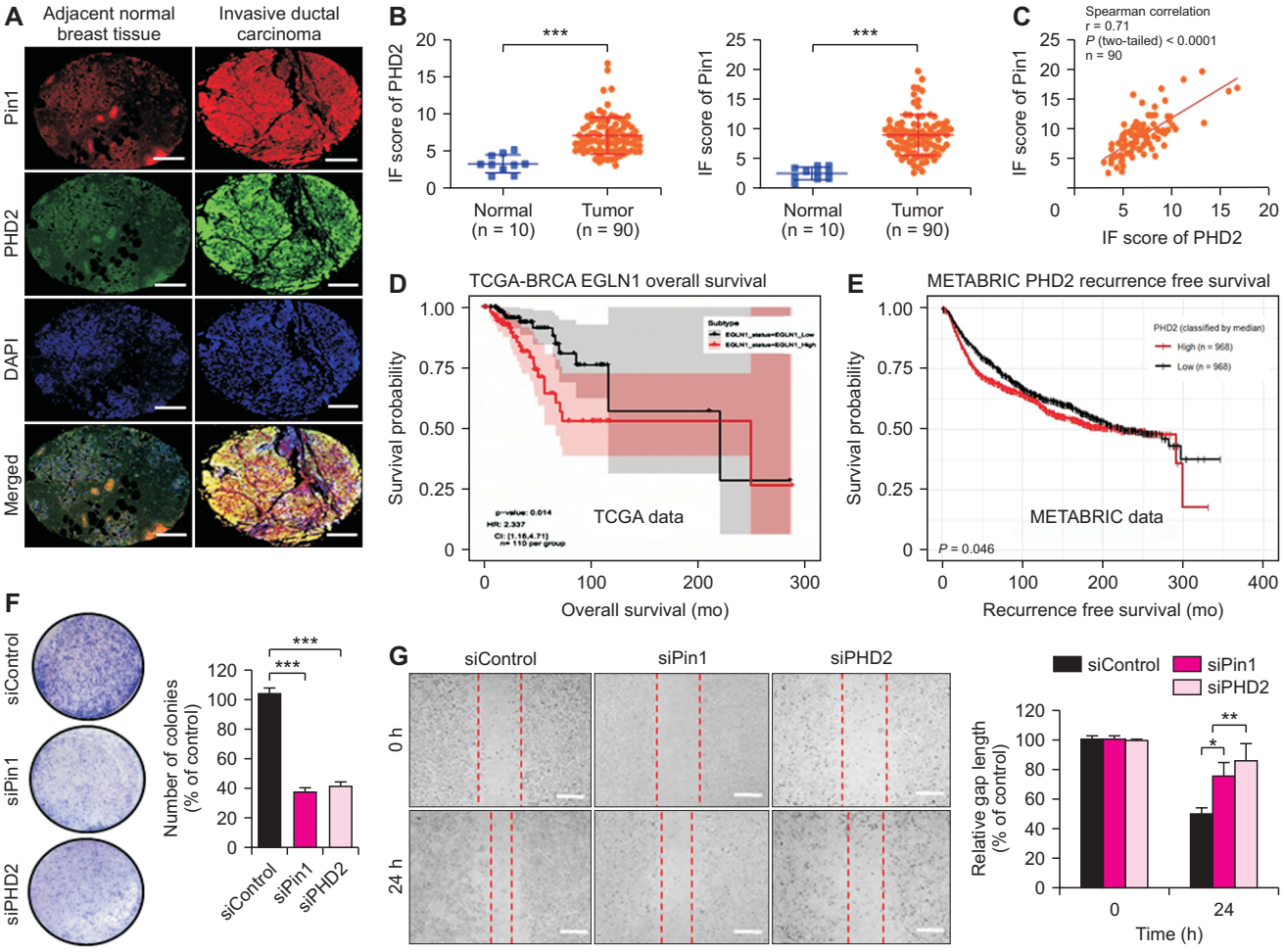


Figure 1. Overexpression of PHD2 and Pin1 and their functional relationship in breast cancer. (A) Representative IF images of Pin1 and PHD2 in breast tumor and surrounding normal tissue arrays. Scale bar, 200 μm. (B) Measurement of Pin1 and PHD2 expression levels based on the tissue microarray IF score. The two-tailed unpaired Student's *t*-test (****P* < 0.001). (C) Spearman analysis of IF data demonstrating a strong, positive correlation between Pin1 and PHD2 (n = 90; r = 0.71). (D) Survival analysis of PHD2- (Egln1) high expression vs. -low expression breast cancer patients in TCGA-BRCA cohort. The grouping was based on log2 (tpm + 0.001) values with the cutoff of 10% and 90% percentiles. The RNA expression and phenotype data was obtained from UCSC Xena Browser (https://xenabrowser.net/datapages/). (E) METABRIC Data-PHD2 Survival analysis by mRNA expression level in breast cancer patients (*P* = 0.046). (F, G) Oncogenic activity of Pin1 and PHD2 in breast cancer cells. (F) Control, Pin1, or PHD2 siRNA was transfected into MCF7 cells in 6-well plates according to the Materials and Methods section. Following crystal violet staining, attached cells were captured on camera, and the percentage of attached cells was determined by counting the number of colonies. Representative sets of photos from three separate experiments are displayed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (****P* < 0.001). (G) After being transfected with siRNA for control, Pin1, or PHD2, MCF7 cells were incubated for 24 hours. Then, using a confocal microscope, cell migration was viewed. The Student's *t*-test was used to establish the statistical significance of the data, which are presented as the mean ± SD (n = 3; **P* < 0.05 and ***P* < 0.01). Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; PHD2, prolyl hydroxylase domain 2; DAPI, 4',6-diamidino-2-phenylindole; IF, immunofluorescence; siRNA, small interfering RNA.

Survival analysis evaluated using the TCGA-BRCA cohort revealed reduction of survival time in patients with high PHD2 (Fig. 1D), and similar results from METABRIC data linked high PHD2 mRNA levels to poor prognosis in breast cancer (Fig. 1E). Further, functional assays demonstrated that silencing Pin1 or PHD2 reduced both clonogenic growth (Fig. 1F) and migration (Fig. 1G) in MCF7 breast cancer cells.

Identification of Pin1 binding sites on PHD2

Pin1 specifically binds to pSer/Thr-Pro motifs on substrate proteins, influencing their conformational, function and stability. PHD2 contains serine and threonine residues with adjacent prolines, forming four potential Pin1 binding sites (pSer/Thr-Pro motifs): S12, S125, S174 (serine residues) and T168 (threonine) as shown in Figure 2A and 2B. liquid chromatography-tandem mass spectrometry (MS) analysis confirmed phosphorylation at S125, T168, and S174 (Fig. 2C). Sequence analysis of PHD2 across multiple species showed conservation of these motifs, with pSer-Pro (pS125-P126 and pS174-P175) and pThr-Pro (pT168-P169) in three species, only pSer-Pro (pS125-P126) in four species, and only pThr-Pro (pT168-P) in five species (Fig. 2D).

After confirming the presence of pSer/Thr-Pro motifs in PHD2, we investigated its interaction with Pin1. Using STRING bioinformatics for curated and experimental databases, an interaction between Pin1 and PHD2 was predicted (Fig. 3A) and subsequently validated by immunoprecipitation. Results showed that Pin1 and PHD2 interact under both normoxic and hypoxic conditions in MDA-MB-231 cells (Fig. 3B) and in MCF7 cells (Figure S1C). Further, co-overexpression of Pin1 and PHD2 in MDA-MB-231 cells demonstrated this physical interaction via PLA fluorescence detection (Fig. 3C).

Nuclear translocation of PHD2 has been shown to be associated with cancer cell growth and tumor-aggressiveness [32-34]. We investigated the intracellular localization of PHD2 and Pin1 in MDA-MB 231 and MCF7 breast cancer cells. Our data show that the majority of PHD2 is localized in the nucleus while Pin1 is detected mainly in the cytoplasm in both cell lines (Fig. 3D and 3E). Western blot analysis of total cell lysates confirmed sustained PHD2 protein expression even in hypoxia (Figure S1D and S1E, Fig. 3E). Notably, hypoxia enhanced the PHD2-Pin1 interaction, primarily the cytoplasm

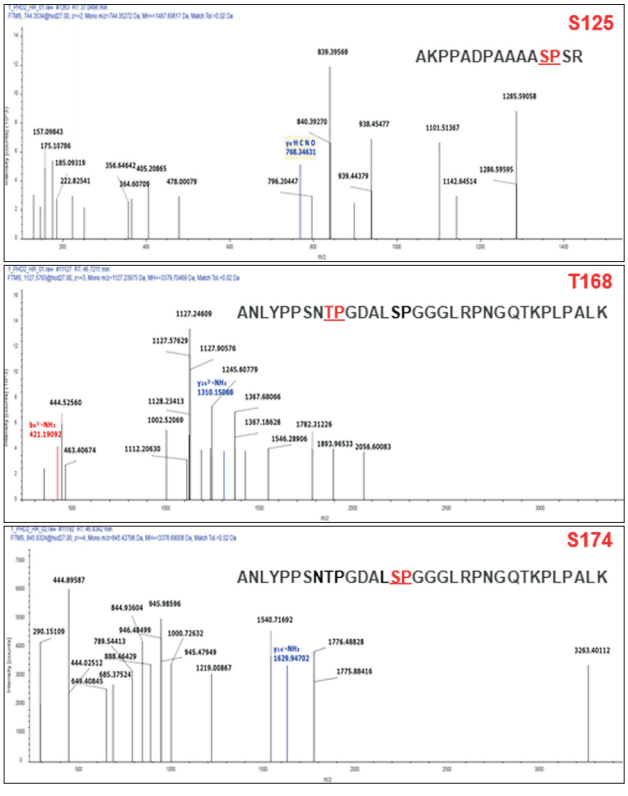
A

MANDSGGPGGPSPSERDRQYCELCGKMENLLRCSRCRSSFYCCKEHQRQDWKKHKLVCQ
GSEGALGHGVGPHQHSGPAPPAVPPPRAGAREPRKAAARRDNASGDAAKGKVKAKPPAD
PAAAASPCRAAAGGQGSAVAAEAEPGKEEPPARSSLFQEKANLYPPSNTPGDALSPGGG
LRPNGQTKPLPALKLALEYIVPCMNKHGICVVDDFLGKETGQQIGDEVRALHDTGKFTD
GQLVSQKSDSSKDIRGDKITWIEGKEPGCETIGLLMSSMDDLIRHCNGKLGSYKINGRT
KAMVACYPGNGTGYVRHVDNPNGDGRCVTCIYYLNKDWDAKVSGGILRIFPEGKAQFAD
IEPKFDRLLFFWSDRRNPHEVQPAYATRYAITVWYFDADERARAKVKYLTGEKGVRVEL
NKPSDSVGKDVF

B

	Position in query protein	Sequence in query protein	Corresponding motif	Corresponding
1	12–13	SP	[pS/pT]P	WW domain binding motif
2	125–126	SP	[pS/pT]P	WW domain binding motif
3	168–169	TP	[pS/pT]P	WW domain binding motif
4	174–175	SP	[pS/pT]P	WW domain binding motif

C



D

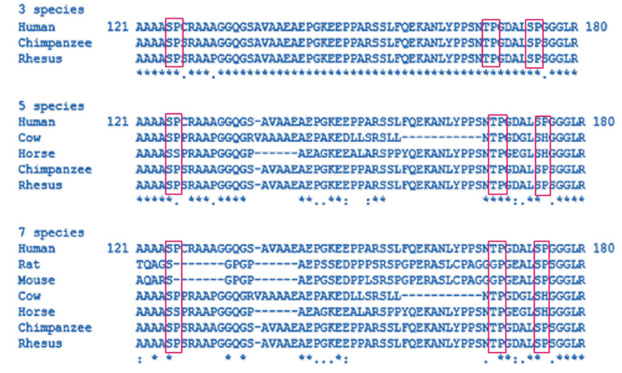


Figure 2. Identification of phosphorylation sites of PHD2. (A) The location of the phosphorylatable serines in PHD2 fragment is shown schematically. (B) PHD2 has the WW domain binding motifs. Four WW binding motifs with the pSer/Thr-Pro sequence are present in the PHD2 protein. Human Protein Reference Database, available at http://www.hprd.org/. (C) The LC-MS/MS analysis was conducted to get the peptide spectra of PHD2. Three residues (S125, T168, and S174) of PHD2 were found to be phosphorylated and recognized as the consensus binding locations for Pin1. (D) PHD2 sequences in S125, T168, and S174 in different species by Multiple Sequence Alignment. PHD2, prolyl hydroxylase domain 2; pSer/Thr-Pro, phospho-serine/threonine-proline; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1.

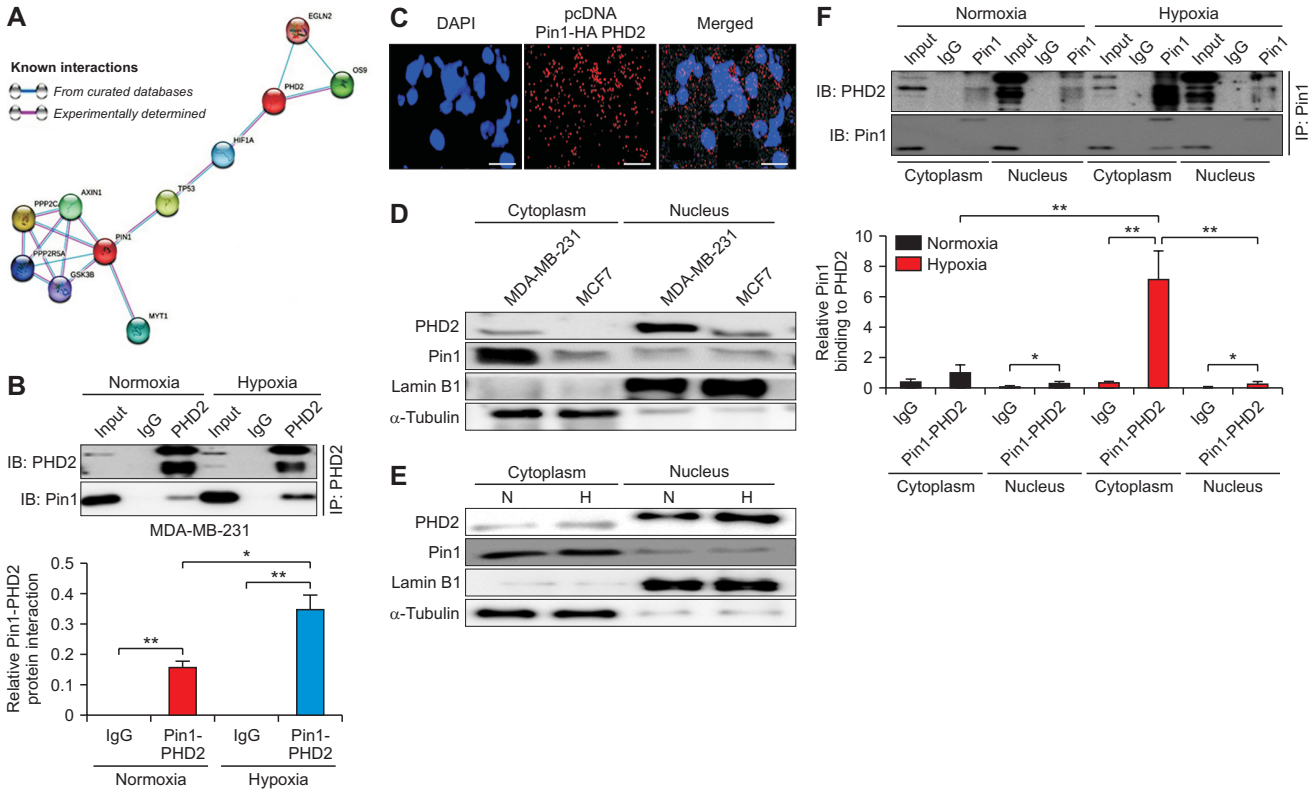


Figure 3. Pin1 and PHD2 interaction in breast cancer cells. (A) Pin1 and PHD2 interaction predicted by the STRING database. (B) The immunoprecipitation technique was used to evaluate how endogenous Pin1 and PHD2 interact in MDA-MB-231 cells in normoxic and hypoxic conditions. Results are expressed as means ± SD (n = 3; *P < 0.05 and **P < 0.01). (C) Interaction between ectopically expressed Pin1 and PHD2 was visualized by the PLA in MDA-MB-231 cells. Corresponding antibodies were used to co-label Pin1 and PHD2. DAPI was used to stain the nuclei. Red dots represent the Pin1 and PHD2 complex. Scale bar, 200 μm. (D) Comparison of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 and MCF7 cells. (E) Localization of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 in normoxic and hypoxic conditions. (F) The endogenous interaction between Pin1 and PHD2 in cytoplasm and nucleus in normoxic and hypoxic conditions. Protein lysates of MDA-MB-231 cells were immunoprecipitated with Pin1 antibody and the proteins were detected with PHD2 and Pin1 antibodies. Results are expressed as means ± SD (n = 3; *P < 0.05 and **P < 0.01). IgG, immunoglobulin G; PHD2, prolyl hydroxylase domain 2; DAPI, 4',6-diamidino-2-phenylindole; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; N, normoxia; H, hypoxia; PLA, proximity ligation assay.

(Fig. 3F).

PHD2 expression regulated by Pin1

In a follow-up experiment, siRNA-mediated knockdown of Pin1 reduced PHD2 expression under both normoxic (Fig. 4A) and hypoxic conditions (Figure S1F), without impacting others PHD isoforms, such as PHD1 and PHD3 (Figure S1G). Pin1 silencing specifically affected PHD2 expression (Fig. 4B), as confirmed by immunofluorescence staining, which showed a marked decrease in PHD2 levels (Fig. 4C). This reduction in PHD2 also weakened the interaction between Pin1 and PHD2 under the same conditions (Fig. 4D). Conversely, knockdown of PHD2 did not impact Pin1 expression levels (Fig. 4E). Since PHD2's primary role involves hydroxylation of HIF, we examined the PHD2-HIF-2α interaction following Pin1 suppression. Notably, Pin1 knockdown via siRNA disrupted the PHD2-HIF-2α interaction in breast cancer cells (Figure S2).

Serine 125 of PHD2 as a critical site for Pin1 binding and oncogenicity

Site-directed mutagenesis studies, in which the serine (S125 and S174) and threonine (T168) residues adjacent to proline were replaced with non-phosphorylatable alanine, revealed that Ser125 is critical for Pin1 binding (Fig. 5A). Additionally, the S125A mutant cells displayed increased PHD2 ubiquitination (Fig. 5B), indicating that PHD2 stabilization relies on the phosphorylation of Ser125 residue (pS125), which Pin1 recognizes.

We then investigated the functional significance of S125A phosphorylation of PHD2 in MDA-MB-231 and MCF7 cells. Cells expressing WT PHD2 and Pin1 exhibited the relatively high clonogenicity in both cell lines (Fig. 5C) and migrative capability in MDA-MB-231 cells (Fig. 5D); these capabilities were attenuated by S125A mutation.

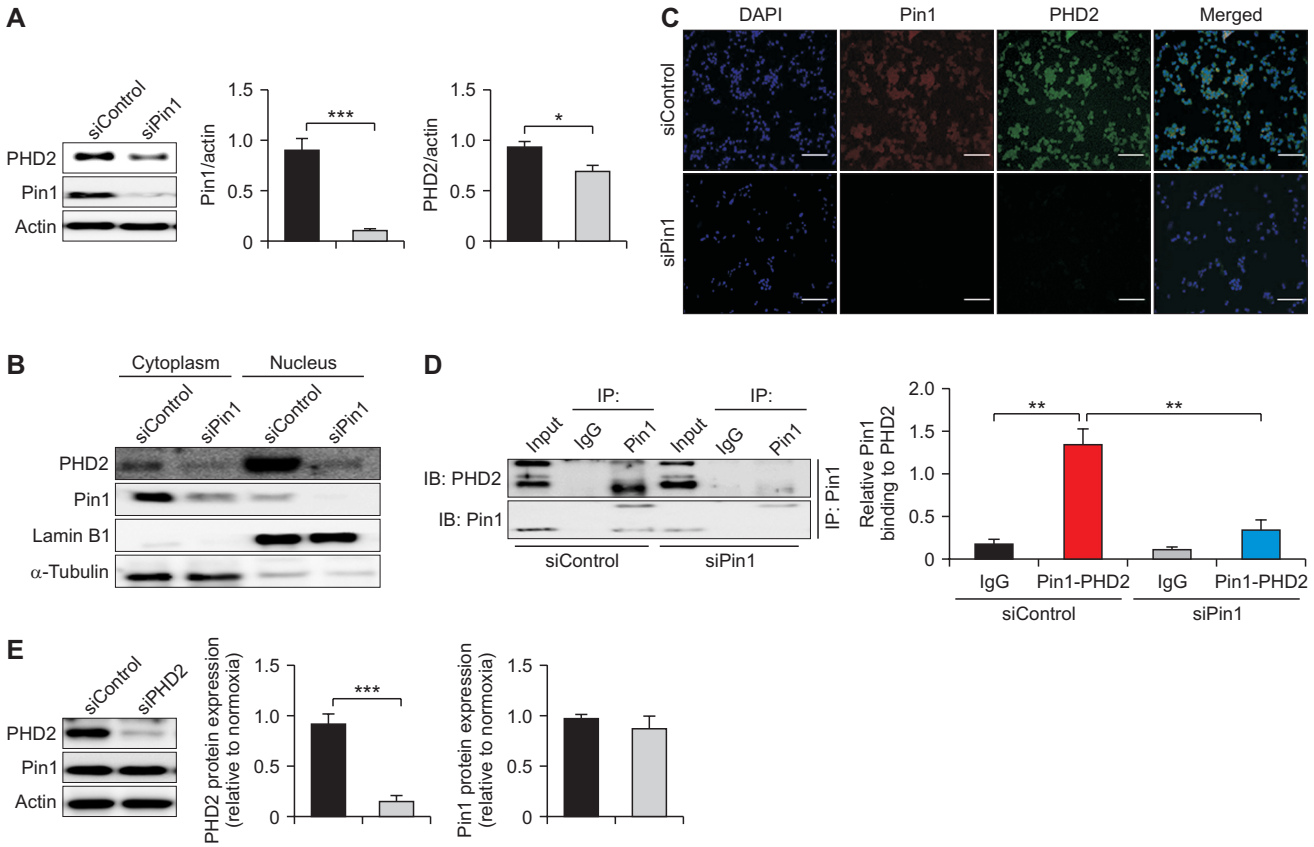


Figure 4. Effects of Pin1 knockdown on expression of PHD2 in breast cancer cells. (A) MDA-MB-231 cells were treated with either control siRNA or Pin1 siRNA. Western blot analysis using anti-Pin1 and anti-PHD2 antibodies was performed on cell lysates (**P* < 0.05 and ****P* < 0.001). (B) Comparison of Pin1 and PHD2 protein expression in cytoplasm and nucleus of MDA-MB-231 transiently transfected with control siRNA and Pin1 siRNA. (C) After Pin1 siRNA was transfected into MDA-MB-231 cells, IF staining was used to evaluate the expression of both Pin1 and PHD2. Scale bar, 200 μm. (D) Interaction of Pin1 with PHD2 in Pin1 knockdown MDA-MB-231 cells confirmed by IF (***P* < 0.01). (E) Protein expression of both Pin1 and PHD2 in MDA-MB-231 cells transfected with PHD2 siRNA. Results are expressed as means ± SD (n = 3; ****P* < 0.001). PHD2, prolyl hydroxylase domain 2; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; DAPI, 4',6-diamidino-2-phenylindole; IgG, immunoglobulin G; siRNA, small interfering RNA; IF, immunofluorescence.

DISCUSSION

Cancer cells frequently encounter low oxygen concentrations (hypoxia) in a tumor microenvironment. To adapt and thrive in these conditions, they upregulate erythropoietin, which promotes red blood cells (erythrocytes) production. The transcription factor HIF-2α plays a pivotal role in erythropoiesis by enhancing the oxygen-carrying capacity of erythrocytes. Under hypoxic conditions in the tumor microenvironment, cancer cells can shift their metabolic focus from mitochondrial respiration to increased glycolysis to sustain ATP production [35]. This metabolic reprogramming is primarily controlled by HIF-1α, which boosts glycolytic enzymes expression. However, recent studies indicate that HIF-2α, activated by abnormal expression of signal-induced proliferation-associated 1, also upregulates multiple glycolysis-related genes in breast cancer cells [36].

To survive in the variable oxygen levels in tumor microenvironment, cancer cells must detect and respond to changes in the oxygen gradient. Significant progress has been made in understanding how hypoxia regulates cellular signaling pathways. The PHD family of enzymes, which require molecular oxygen for their catalytic activity, are key candidates for oxygen sensing [37]. The PHD family consists of three proteins-PHD1, PHD2 and PHD3- that, while structurally similar, exhibit distinct catalytic activities and functions [38-40]. Although PHDs are well-known for their role in regulating the stability of HIF-2α and HIF-1α under normoxic conditions, their precise functions in the hypoxic tumor microenvironment remain unclear. These proteins use oxygen and 2-oxoglutarate as substrates, with Fe (II) and ascorbate as cofactors, to catalyze the oxidation of conserved proline residues in HIF proteins, acting as the primary oxygen sensors within cells [41,42].

Data regarding functions of PHD2 in various types of cancer are conflicting and discordant, while some studies suggest tumor promoting effects while others indicating tumor suppressive effects exerted by this monooxygenase [43]. Beyond its direct involvement in regulation cancer cell prolifera-

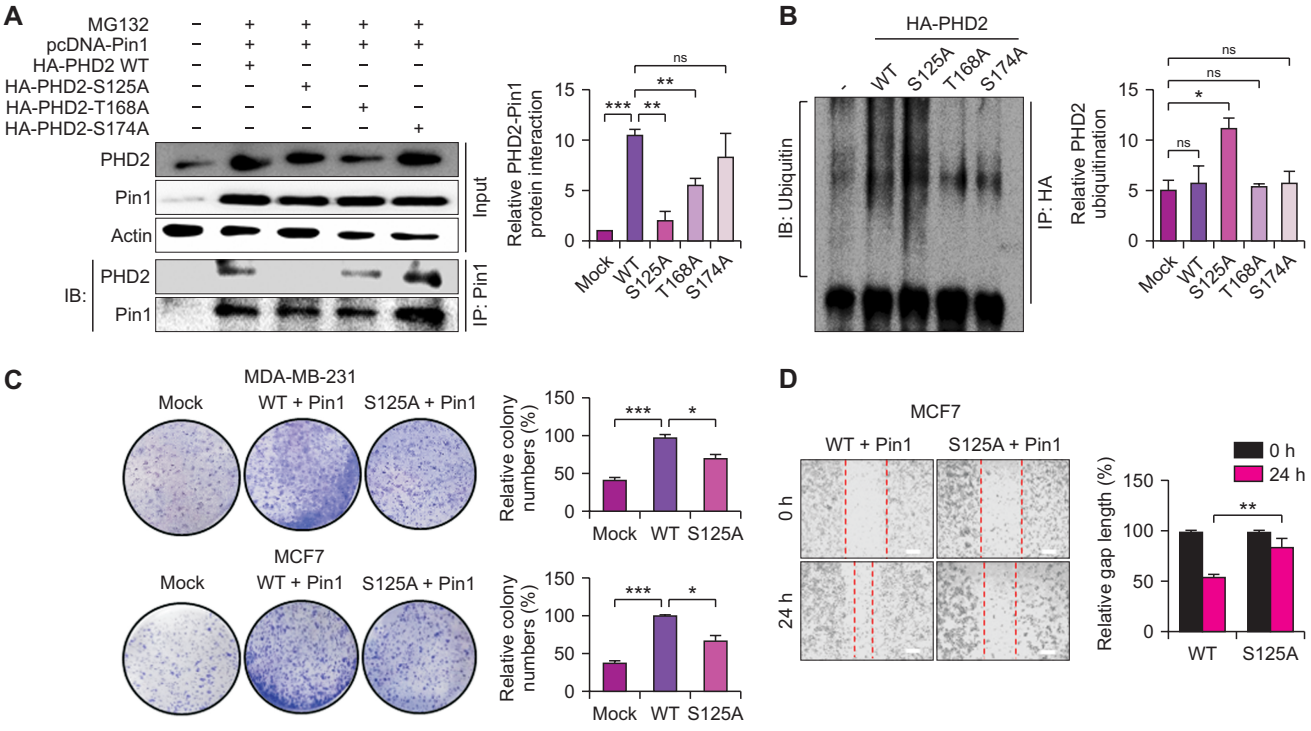


Figure 5. The precise sites of PHD2 (S125, T168, and S174) involved in Pin1 binding. (A) PHD2-Pin1 interaction was compared in WT and mutant cells in which a particular serine was changed into an alanine. pcDNA-Pin1 and HA-tagged WT PHD2 or the corresponding mutant constructs were co-transfected into HEK293T cells, and the cell lysates were then subjected to IP analysis. Quantitative analysis of the interaction between Pin1 with WT or non-phosphorylatable mutants (PHD2 S125, T168, and S174; $^{**}P < 0.01$ and $^{***}P < 0.001$). (B) Ub-PHD2 in HEK293T cells was measured by IP of HA, followed by a Western blot experiment using an anti-ubiquitin antibody ($^{*}P < 0.05$). (C) MDA-MB-231 and MCF7 cells were co-transfected with pcDNA-Pin1 and HA-tagged WT PHD2 or mutant forms (S125A, T168A, and S174A). The cells were stained with crystal violet and then photographed ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (D) MCF7 cells in 4-well plates were co-transfected with pcDNA-Pin1 and HA-tagged WT PHD2 or mutant forms (S125A, T168A, and S174A) for the cell migration assay. The results are presented as the mean ± SD (n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). PHD2, prolyl hydroxylase domain 2; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; WT, wild type; Ub-PHD2, ubiquitinylated PHD2; IP, immunoprecipitation; ns, not significant.

tion and growth, PHD2 has also been shown to play a critical role in tumor vascularization [10] and the tumor microenvironment [7], contributing to cancer progression. The expression of PHD isoforms varies significantly between cancerous and adjacent normal tissues. Notably, PHD2 is overexpressed in several types of human cancers including lung, liver, kidney, and breast cancers [7,44], which aligns with our recent findings showing its pro-tumorigenic role in breast cancer. However, in colorectal cancer, low levels of PHD2 expression are linked to poorer prognosis [45].

The primary role of PHD2 in the tumor microenvironment has traditionally been linked to its regulation of HIF protein levels in hypoxic cancer cells compared to the surrounding normoxic normal cells. However, emerging research is revealing several non-canonical functions of PHD2 that are independent of its HIF hydroxylation activity. For instance, PHD2 has been shown to negatively regulate NF-κB signaling, regardless of its ability to hydroxylate HIF [8]. Additionally, PHD2's binding to EGFR is essential for maintaining EGFR stability and activating the extracellular-signal-regulated kinase and PI3K pathways, which are critical for cancer cell growth and survival [12]. While PHDs require oxygen for optimal function, it does not necessarily imply that oxygen levels directly affect their catalytic activity. In fact, PHDs may still function within the hypoxic tumor microenvironment, where oxygen is present at relatively low concentrations (pseudohypoxia).

Phosphorylation is a key mechanism that regulates the activity of many enzyme systems. However, phosphorylation alone is not sufficient to control PHD function. While phosphorylation is necessary, other factors may also be involved in its regulation. It is suggested that hypoxia may increase reactive oxygen species production, which in turn could activate a series of kinases. Several proteins have been identified that regulate PHD2 expression through phosphorylation including hypoxia-regulated proteins such as mTOR, p70S6K, and GSK3β which phosphorylate PHD2 [46,47]. PHD2 consists of 426 amino acid residues, forming a long intrinsically disordered N-terminal region spanning residues 1 to 187, followed by a well-structured oxygenase domain that serves as the catalytic center (residues 188 to 418) [47-49]. The phosphorylation of Ser/Thr residues preceding the proline residue and

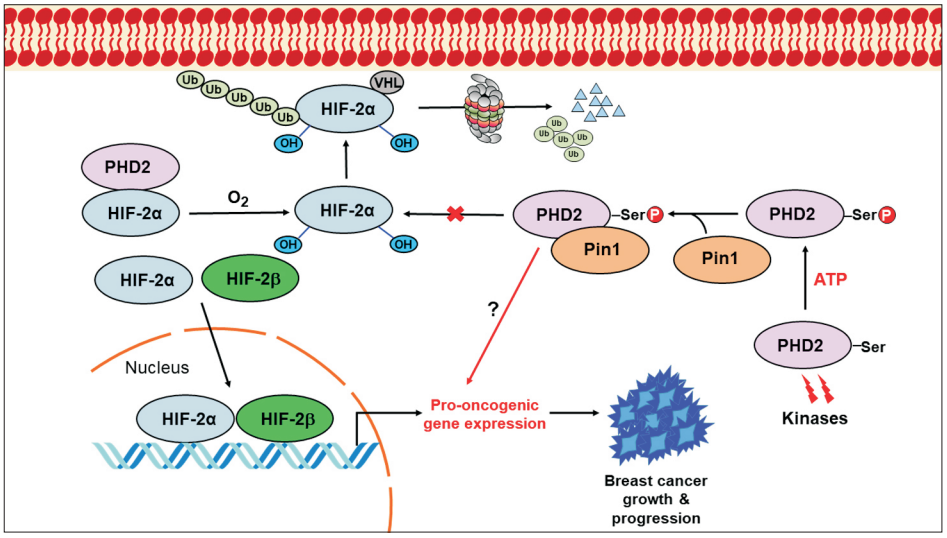


Figure 6. A proposed model for the regulation of PHD2 by Pin1 in breast cancer. PHD2 can act in normoxia and hypoxia. PHD2 undergoes phosphorylation at a specific Ser residue (e.g., S125), which facilitates its interaction with Pin1. As a result, PHD2 is unable to hydroxylate HIF-2α and translocates to the nucleus where it might be involved in the proliferation of breast cancer. PHD2, prolyl hydroxylase domain 2; HIF, hypoxia-inducible factor; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; Ser, serine.

subsequent Pin1-mediated proline isomerization serve as a regulatory mechanism for numerous oncogenic and tumor suppressor proteins [50-52].

It has been reported that PHD2 is primarily localized in the cytoplasm [43]. However, PHD2 is capable of shuttling between the cytoplasm and the nucleus. Notably, PHD2 protein expression is predominantly found in the nucleus of tumor tissues [38], with nuclear localization being more pronounced than cytosolic localization under hypoxic conditions [9]. In line with these observations, our study shows that in MDA-MB-231 and MCF7 human breast cancer cells, PHD2 expression is higher in nucleus compared to the cytoplasm, with an increase under hypoxic conditions. Similar results were observed in U-2OS cells, where strong nuclear expression of PHD2 was evident in hypoxic conditions, as well as following NO treatment [53].

In the present study, we identified specific serine and threonine residues in PHD2 that are essential for its interaction with Pin1. Previous research in colon cancer has suggested that PHD2 can function in an oxygen-independent manner through post-translational modification [9]. While PHD2 is the key regulator of HIF-1α stability, its phosphorylation status plays a role in modulating HIF-1α hydroxylation [42]. Specifically, Ser125 in PHD2 has been reported to be phosphorylated by various kinases [9], and this phosphorylation can regulate PHD2 activity, though it does not affect its interaction with HIF-1α. Furthermore, PHD2 can be activated through phosphorylation of Ser125, a process catalyzed by the mTOR pathway, particularly by P70S6K [9]. Our findings highlight the critical role of Ser125 in stabilizing PHD2 through its interaction with Pin1. While other serine residues, such as Ser12 and Ser14, have been shown to be phosphorylated, their precise role in regulating PHD2 activity remains unclear [9].

A salient finding of our research is the role of nuclear oncogenic PHD2 in influencing breast cancer progression. The activity of PHD2 is regulated by various signaling pathways, many of which are linked to oxygen deprivation. We observed a strong association between PHD2 and Pin1 in breast cancer tissues. A model for the regulation of PHD2 by Pin1 in breast cancer is proposed (Fig. 6). It is noticeable that hypoxia upregulates the PHD2-Pin1 interaction. Aberrant PHD2 overexpression is associated with adverse outcomes in breast cancer which could potentially increase its aggressiveness and mesenchymal transition. PHD2 holds promise as a therapeutic target for breast cancer treatment, particularly considering its pro-oncogenic role in influencing erythropoietin production and tumor growth. Moreover, PHD2 inhibitors could be tested in clinical studies for their potential application in the management of cancer and other disorders.

FUNDING

Grants were used to fund this work. The National Research Foundation of the Republic of Korea provided funding for this study through the BK21 FOUR Program (5120200513755 to Y. K. Shin) and the Basic Science Research Grant (2021R1A2C2014186 to Y.-J. Surh).

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.24.031.

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