Prolyl hydroxylase 2 inhibits glycolytic activity in colorectal cancer via the NF-κB signaling pathway

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Abstract. A variety of malignancies preferentially meet energy demands through the glycolytic pathway. Hypoxia-induced cancer cell adaptations are essential for tumor development. However, in cancerous glycolysis, the functional importance and underlying molecular mechanism of prolyl hydroxylase domain protein 2 (PHD2) have not been fully elucidated. Gain- and loss-of-function assays were conducted to evaluate PHD2 functions in colon cancer cells. Glucose uptake, lactate production and intracellular adenosine-5'-triphosphate/adenosine diphosphate ratio were measured to determine glycolytic activities. Protein and gene expression levels were measured by western blot analysis and reverse transcription-quantitative PCR, respectively. The human colon cancer xenograft model was used to confirm the role of PHD2 in tumor progression *in vivo*. Functionally, the data demonstrated that PHD2 knockdown leads to increased glycolysis, while PHD2 overexpression resulted in suppressed glycolysis in colorectal cancer cells. In addition, the glycolytic activity was enhanced without PHD2 and normalized after PHD2 reconstitution. PHD2 was shown to inhibit colorectal tumor growth, suppress cancer cell proliferation and improve tumor-bearing mice survival *in vivo*. Mechanically, it was found that PHD2 inhibits the expression of critical glycolytic enzymes (glucose transporter 1, hexokinase 2 and phosphoinositide-dependent protein kinase 1). In addition, PHD2 inhibited Ikkβ-mediated NF-κB activation in a hypoxia-inducible factor-1α-independent manner. In conclusion, the data demonstrated that PHD2/Ikkβ/NF-κB signaling has critical roles in regulating glycolysis and suggests that PHD2 potentially suppresses colorectal cancer.

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Introduction

Unlike non-malignant cells, cancer cells utilize a different program for glucose metabolism. Due to the altered tumor microenvironment, cancer cells have to elevate glycolytic activity to generate adequate adenosine-5'-triphosphate (ATP) within a hypoxic microenvironment to maintain rapid cell proliferation. This phenomenon of continuously producing high lactate in cancer cells in the presence of oxygen is known as the Warburg effect (1-3).

During tumor expansion, uncontrollable cell proliferation and abnormal tumor angiogenesis impede the nutrient supply for cancer cells (4). Consequently, hypoxic regions, where oxygen concentration is low, are frequently found in solid tumors. To adapt to this hypoxic environment, tumor cells usually adopt glycolysis for their energy supply to facilitate angiogenesis, which, in turn, contributes to the continued growth and survival of a tumor (5). Therefore, oxygen homeostasis and regulating downstream cellular metabolic events are critical during tumor progression. Members from the family of prolyl hydroxylase (PHD), a group of well-established oxygen sensors, are potentially the major participants that sense oxygen and regulate glucose metabolism in tumors (6-8).

PHDs mediate hypoxia-inducible factor-1α (HIF-1α) hydroxylation at 2 prolyl residues in the oxygen-dependent degradation domain, enabling it to be captured, ubiquitinated and ultimately degraded in the proteasome (9). There are three known PHD isoforms in humans; PHD1, PHD2 and PHD3. PHD2 is one of the key enzymes mediating HIF-1α degradation under normoxia (10-12). In addition, PHD2 is downregulated or even barely detected in numerous cancer cells and tumor tissues (13). The decreased expression of PHD2 is significantly correlated with some genetic disorders, which ultimately develop malignancies (12). Therefore, such findings render PHD2 a potential tumor suppressor (14). In addition, PHD2 has been associated with tumor vasculature, which occurs after tumor glycolysis (15-17). Nevertheless, the role of PHD2 in tumor glycolysis is still unknown. The present study focused on investigating glycolysis, including glucose uptake,

lactate production and energetic molecular quantification and confirming the regulatory effect of PHD2 in glycolysis in colon cancer cells by gain- and loss-of-functional assays.

Materials and methods

Cell lines. HEK293T and human colorectal carcinoma cell lines (HT-29, HCT116, Ls174t, LoVo, SW480, SW620, RKO and Caco-2) were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37˚C in an atmosphere containing 5% CO_2. All reagents for cell culturing were purchased from Invitrogen (Thermo Fisher Scientific, Inc.).

PHD2 knockdown and overexpression. For PHD2 silencing, the pGCL-GFP expressing shRNA against PHD2 [short hairpin (sh)PHD2; target sequence: GGATGGAATCCATGA GCTA] or non-targeting shRNA (Mock; TTCTCCGAACGT GTCACGT) were designed and synthesized by Shanghai GeneChem Co., Ltd. For PHD2 overexpression, the open reading frame of PHD2 was constructed into the pGC-FU lentiviral vector (pGC-FU-PHD2) (Shanghai GeneChem Co., Ltd.), while the empty vector was used as vehicle control (vehicle).

HEK293T cells were co-transfected with helper plasmids (pHelper 1.0 and pHelper 2.0; Shanghai GeneChem Co., Ltd.) and lentiviral plasmids (pGCL-GFP-shPHD2 or pGC-FU-PHD2) using Lipofectamine®2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 24 h for lentivirus production. Lentivirus was harvested at 48 and 72 h following transfection. Cell debris was removed by centrifugation (300 x g; 10 min; room temperature) and the virus was then filtered through a 0.45 μm cellulose acetate filter and collected by ultracentrifugation (100,000 x g; 4 h; 4˚C).

For lentivirus infection (37˚C for 12 h), Ls174t and SW480 cells were transduced with PHD2 silencing (PHD2-KD), overexpressing (PHD2-OE) lentivirus, or control lentivirus (represented as MOCK for non-targeting shRNA and vehicle for empty vector), separately, at the multiplicity of infection of 10 (Ls174t) or 20 (SW480). At four days following infection, GFP-positive cells were isolated by a FACSCalibur flow cytometer (BD Biosciences). PHD2 overexpression and silencing were confirmed by western blotting.

HIF-1α lentiviruses transduction. For HIF-1α silencing, the pLKO.1-puro lentiviral vectors encoding shRNA targeting HIF-1α (HIF-1α-KD) or non-targeting control shRNA (MOCK; MilliporeSigma) were obtained for lentiviruses packaging. The cells were transduced with viral supernatant containing 8 μg/ml of Polybrene (MilliporeSigma) and then selected with medium containing 0.6 μg/ml of puromycin (MilliporeSigma) after 12 h (18).

Reverse transcription-quantitative (RT-q) PCR. Human cancerous and adjacent normal colorectum were collected from specimens directly after surgery. Total RNA was extracted using the TRIzol® (Thermo Fisher Scientific, Inc.) method and the complementary DNA was synthesized using a commercially available kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RT-qPCR was carried out using an iCycler system (Bio-Rad Laboratories, Inc.) with SYBR® Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR amplification was performed with the following cycling conditions: i) Initial activation at 95˚C for 10 min, and subsequently 40 cycles of denaturation at 95˚C for 15 sec; ii) Annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec (40 cycles). Primers used were as follows: Phd2: 5'-CGATCCGGTGAC TTTTCCCAC-3' and 5'-GTAGGAGGTCTCCGTCCT-3'; hexokinease-2 (Hk2): 5'-AAGATGCTGCCCACCTACG-3' and 5'-TCGCTTCCCATTCCTCACA-3'; PHD kinase 1 (Pdk1): 5'-AAGATGAGTGACCGAGGAGGT-3' and 5'-CCA TAACCAAAACCAGCCAGAG-3'; glucose transporter 1 (Glut1): 5'-GTGCTCCTGGTTCTGTTCTTCA-3' and 5'-GCC AGAAGCAATCTCATCGAA-3'; β-actin: 5'-ACCCCGTGC TGCTGACCGAG-3' and 5'-TCCCGGCCAGCCAGGTCC A-3'. The expression levels of detected genes were normalized to the level of β-actin mRNA. The relative expression of the target genes was calculated using the following formula: gene expression=$2^{-(\Delta Cq\text{-normal}-\Delta Cq\text{-tumor})}$ (19). The experiment was independently repeated three times.

ELISA assay. Cells were washed with ice-cold PBS, detached and pelleted by centrifugation (1,000 x g; 5 min; 4˚C). The cells were resuspended in 300 μl of hypotonic buffer (10 mM Hepes; pH 7.6; 60 mM KCl; 1 mM EDTA; 1 mM dithiothreitol) containing phenylmethylsulfonyl fluoride (PMSF; 1 mM) and protease inhibitor cocktail (10 μl/ml) and incubated on ice for 15 min. Then, 20 μl of 10% NP-40 was added and the tube was vortexed for 10 sec. The lysates were cleared by centrifugation (13,000 x g; 1 min; 4˚C) and supernatants (cytosolic fractions) were collected and stored at -80˚C.

Nuclear contents were obtained from the pellets. First, the pellets were resuspended in extraction buffer (20 mM Tris-HCl; pH 8; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA) supplemented with PMSF (0.5 mM), protease inhibitors cocktail (10 μl/ml) and glycerol (25% v/v). After 30 min at 4˚C, the debris was removed by centrifugation (13,000 x g; 15 min; 4˚C). The supernatant containing nuclear proteins was aliquoted and stored at -80˚C for further analysis. Protein concentrations were determined using a Bradford protein assay (Bio-Rad Laboratories, Inc.).

For the p50 detection, the levels of p50 were measured using a TransAM NF-κB p50 ELISA kit (cat. no. 31101; Active Motif, Inc.) according to the manufacturer's instructions with a microplate reader (Bio-Rad Laboratories, Inc.). The experiment was conducted in triplicates and was independently repeated three times.

Western blotting and co-immunoprecipitation. Western blotting was conducted using lysates extracted from human tissues or cells as previously described (20). In general, tissues or cells were solubilized in RIPA buffer (BioTeke Corporation) containing a proteinase inhibitor cocktail (1 mM EDTA; 1 mM PMSF; 1 mM iodoacetic acid). After the protein concentration was determined with the Bradford method, identical amounts of proteins (50 μg) were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene

fluoride membranes. The membranes were then blocked with 5% skimmed milk for 2 h at room temperature followed by overnight incubation with antibodies against PHD2 (1:2,000; cat. no. NBP1-30328; Novus Biologicals, LLC), glucose transporter 1 (GLUT1; 1:500; cat. no. sc-377228; Santa Cruz Biotechnology, Inc.), PDK1 (1:500; cat. no. sc-515944; Santa Cruz Biotechnology, Inc.), hexokinase (HK) 2 (1:1,000; cat. no. sc-374091; Santa Cruz Biotechnology, Inc.), p65 (1:1,000; cat. no. sc-8008; Santa Cruz Biotechnology, Inc.) and GAPDH (1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) at 4˚C. The following day, membranes were rinsed and incubated with HRP-conjugated secondary antibodies (1:2,000, cat. no. BA1082; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce; Thermo Fisher Scientific, Inc.) was used for development and images were captured using a Gel Doc XR system (Bio-Rad Laboratories, Inc.). β-actin was used as the loading control. ImageJ software (NIH; version 1.8.0) was used for densitometry.

For immunoprecipitation assay, fresh cell lysates using RIPA buffer (cat. no. PP1901; BioTek Instruments, Inc.) containing a proteinase inhibitor cocktail (1 mM EDTA; 1 mM PMSF; 1 mM iodoacetic acid) (500 μg) were incubated with primary antibody (1 μg) at 4˚C for 3 h. Then, 20 μl of protein A/G PLUS agarose beads (cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) was supplemented for overnight incubation. The following day, the beads were pelleted and collected by centrifugation (10,000 x g; 15 sec; 4˚C), followed by washing with RIPA buffer as aforementioned. Then, immobilized proteins were eluted in the boiled SDS buffer for electrophoresis and immunoblotting. Primary antibodies were used to detect the HA-tag (1:1,000; cat. no. sc-805; Santa Cruz Biotechnology, Inc.), Flag-tag (1:4,000; cat. no. F3165; MilliporeSigma), IKKα (1:1,000; cat. no. 2682; Cell Signaling Technology, Inc.), anti-IKKβ (1:1,000; cat. no. 2684; Cell Signaling Technology, Inc.). HRP-conjugated anti-rabbit (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc.), -mouse (1:5,000; cat. no. 7076; Cell Signaling Technology, Inc.), or -goat (1:5,000; cat. no. 7075; Cell Signaling Technology, Inc.) secondary antibodies were used for incubation for 1 h at room temperature.

Electrophoretic mobility shift assay. An oligonucleotide containing the IκB-binding site was radiolabeled with [γ-^{32}p] dATP using T4 polynucleotide kinase at 37˚C for 1 h. The labeled oligonucleotide was purified with the QIAquick Nucleotide Removal Kit (cat. no. 28304; Qiagen GmbH) according to the manufacturer's instruction. The binding between probe (1 ng) and nuclear extracts (5 ng) was allowed to form in 20 μl reaction buffer (1 μg poly (dI-dC); 0.1 μg BSA; 1 mM DTT; 60 mM KCl; 10% glycerol and 20 mM HEPES, pH 8.4) at 30˚C for 30 min. Then, 1 μg of p65 antibody (cat. no. 05-361; Upstate Biotechnology, Inc.) was added to immobilize the oligonucleotide/protein complexes, which were then separated by native electrophoresis in a 6% polyacrylamide gel that was exposed to an X-ray film (Amersham; Cytiva) for 96 h at -80˚C.

2-deoxy-D-glucose uptake assay. Cells (1x10^7 cells/ml) were washed twice with PBS and resuspended and incubated in 100 μl PBS supplemented with 5 mM of 2-deoxy-D-glucose [containing 2 μCi/ml 2-deoxy-(1-^{3}H) glucose] at 37˚C for 30 min. The glucose uptake was terminated by adding 100 μl of ice-cold PBS containing 100 μM phloretin. The cells were subjected to brief centrifugation (20,000 x g; 30 sec; room temperature). Then, the cell pellets were washed once and transferred into a scintillation vial containing 10 ml of Optifluor (Perkin-Elmer Inc.). The incorporated radioactivity was measured by a liquid scintillation counter (Beckman Coulter, Inc.). The uptaken 2-deoxy-[1-3H] glucose concentrations were presented as nmol/10^4 cells.

L-lactate assay. The day before the assay, cells were seeded (1.2x10^5 per well) in 12-well plates in complete medium in quadruplicate. The medium was replaced with starve medium (DMEM containing 2% FBS) on the next day for 48 h. Then, the medium was collected and the lactate concentration was determined using a fluorometric lactate assay kit (cat. no. K607; BioVision, Inc.). Finally, the results were normalized to protein content.

Measurement of intracellular adenine nucleotide levels. For ATP and adenosine diphosphate (ADP) measurement, cell lysate samples were deproteinized with 5% trichloroacetic acid at various times before and after the glucose addition. Following centrifugation (14,000 x g; 10 sec; 4˚C), the supernatants were neutralized by ether extraction, lyophilized and stored at -80˚C until further analysis. Then, the nucleotide extracts were analyzed by high-performance liquid chromatography using an anion exchange hypersil C18 column (4.6x250 mm) at a flow rate of 0.9 ml/min over a 10-min buffer gradient (0.1 mol/l KH_2PO_4, pH 6.0). The eluting flow was continuously monitored by UV absorption at 254 nm and the peaks of nucleoside triphosphates were quantified by electronic integration with external standards used as reference. Intracellular ATP contents were quantified and normalized to cell number. The results are presented as the ATP/ADP ratio, which is unaffected by sample volume loss during ether extraction or cell numbers.

Human colorectal cancer xenografts in immunodeficient mice. A total of 48 combined immunodeficient (SCID) mice (male, 6-8 weeks old, 20-25 g) were purchased from the Army Medical University Institute of Experimental Animal. Mice were fed at an ambient of 45-55% relative humidity with the constant temperature of 21±2˚C under a 12-h light/dark cycle. Lentivirus for PHD2-silencing or -overexpression and their corresponding controls were used to transduce SW480 (for PHD2 overexpression) or Ls174t cells (for PHD2 depletion). SCID mice were randomly divided into 3 groups (n=16) for PHD2 overexpression, silencing and control. Stably transduced SW480 and Ls174t cells or the corresponding parental cells (~2x10^6) in 200 μl of PBS were admixed with 200 μl of Matrigel (BD Biosciences). The cells were delivered to the mice through subcutaneous injection. At three days after injection, the mice were subjected to measurements of tumor size, body weight and other side effects twice a week. Tumor volume (mm^3) was calculated by the formula: 0.52 x length x width x thickness. After 40 days, mice were sacrificed (n=6) and tumors were excised for further analysis. The rest of the

mice were subjected to monitoring until they reached the humane endpoint for survival analysis. The humane endpoint was defined as weight loss of more than 20% and mice were sacrificed by CO_2 asphyxiation (at the rate of 30-70% of the chamber volume per minute) followed by cervical dislocation. Survival rate was analyzed using the Kaplan-Meier method. Animal studies were performed under a protocol approved by the Army Medical University Institutional Animal Care and Use Committee (approval no. AMUWEC20232707).

Ki-67 proliferation index. Tumor tissues harvested from the xenografts were fixed in 10% formalin for 24 h at room temperature, gradual dehydration in increasing alcohol concentrations and then embedded within paraffin for 8 h at room temperature as previously described (21). Tissue sections were then incubated with antibodies against Ki67 (1:100; cat. no. NB500-170; Novus Biologicals, LLC). Nuclei were counter-stained with Mayer's hematoxylin (MilliporeSigma) for 5 min at room temperature. Ki-67-positive cell from 6-8 random views of each section was counted and images captured under a light inverted microscope (XSZ-D2; Olympus Corporation) by a researcher who was blinded to this study. ImageJ software (NIH; v1.8.0) was used to process the results and the data were presented as the average percentage of Ki-67-positive cells per view (22).

Statistical analysis. The results were analyzed using SPSS 13.0 software (v13.0; SPSS, Inc.). One-way analysis of variance with Tukey's post hoc test was performed to compare results from different groups. The quantitative data were expressed as the mean ± SEM. All experiments were repeated three to five times independently. The survival analysis was performed using the log-rank test. Pearson's correlation coefficient was used to evaluate the relationship between the expression of PHD2 and glycolysis-related genes in cancerous and adjacent normal colon tissues. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PHD2 is decreased in colorectal tumors. A total of 45 paired human colorectal tumors and adjacent normal tissues were interrogated for PHD2 expression. PHD2 reduction was observed in 29 of 45 (64.4%) tumors compared with the paired normal colorectal tissues (Fig. 1A). In addition, univariate analysis revealed that PHD2 expression is significantly different in tumor and paired normal tissues at the transcriptional level ($P<0.001$). Notably, our previous study demonstrated that the decreased PHD2 expression in tumor tissue favors higher tumor grades and poorer overall survival of colorectal cancer patients (23). In agreement with the findings at the mRNA level, decreased PHD2 expression at the protein level was also observed in all tested paired samples (Fig. 1B). In addition, results from our cell-based immunoblotting assay also revealed that PHD2 was barely detected in most tested colon cancer cells, except for Ls174t cells (Fig. 1C).

Low expression levels of PHD2 correlate with high expression levels of glycolysis-related genes. Genes closely related to glycolysis, such as HK2, PDK1 and GLUT1, were also evaluated in tumor and paired normal adjacent tissues. Consistent with previous reports (24-26), the mRNA levels of those genes, including HK2 ($P<0.01$), PDK1 ($P<0.05$) and GLUT1 ($P<0.001$), were significantly higher in tumor tissues than in paired normal tissues (Fig. 2A). To elucidate the potential relationships between the expression levels of PHD2 and glycolysis-related genes, we plotted PHD2 mRNA levels against HK2, PDK1 and GLUT1 mRNA levels in colorectal tissues from both tumor and adjacent normal colorectum. As a result, the expression of HK2 ($P<0.00001$), PDK1 ($P=0.00217$) and GLUT1 ($P<0.0019$) were significantly inversely-correlated with that of PHD2 in colorectal tumors (Fig. 2B). Together, these findings suggested that PHD2 is downregulated in colorectal tumor tissues and may be involved in tumor-associated glycolysis.

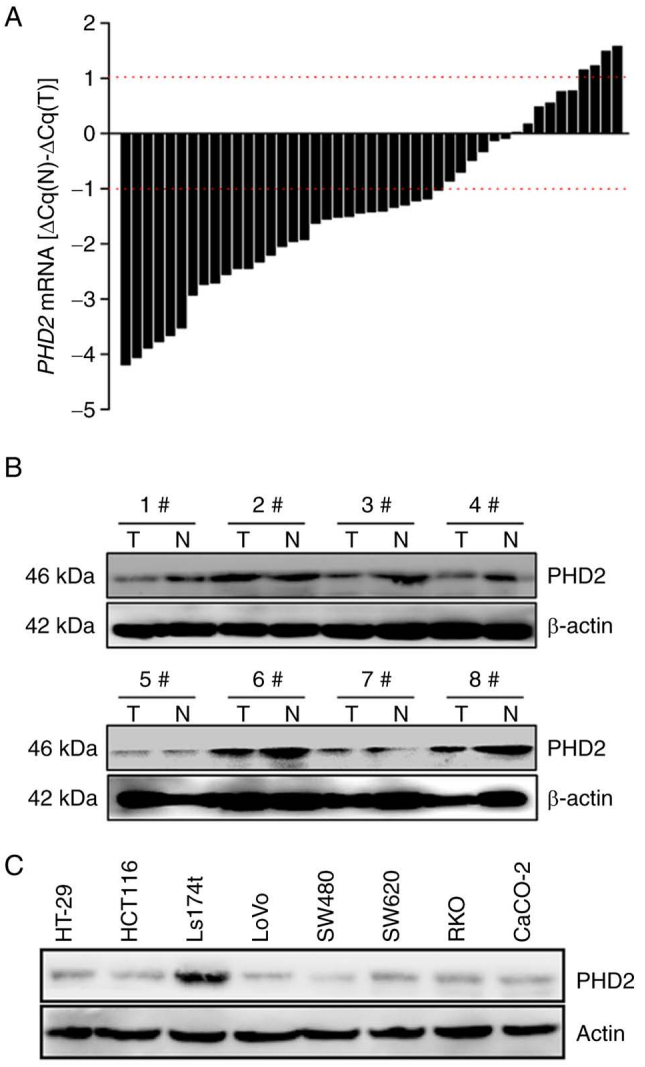

Figure 1. Evaluation of PHD2 expression in patient samples and colorectal cancer cell lines. (A) Results of RT-qPCR detecting PHD2 mRNA level. Δ*Cq(T)*: GAPDH Cq value subtracted from PHD2 Cq value in normal tissue [Δ*Cq(N)*] or tumor [Δ*Cq(T)*]. Bar value: (Δ*Cq(N)*-Δ*Cq(T)*) value indicated the PHD2 mRNA difference in normal tissue and paired tumor and the differences are expressed in log2 scale. (B) Determination of PHD2 protein level in human samples. (C) Determination of PHD2 expression in human colorectal cancer cell lines. T, tumor tissue; N, paired adjacent normal tissue; PHD2, prolyl hydroxylase domain protein 2; RT-qPCR, reverse transcription-quantitative PCR.

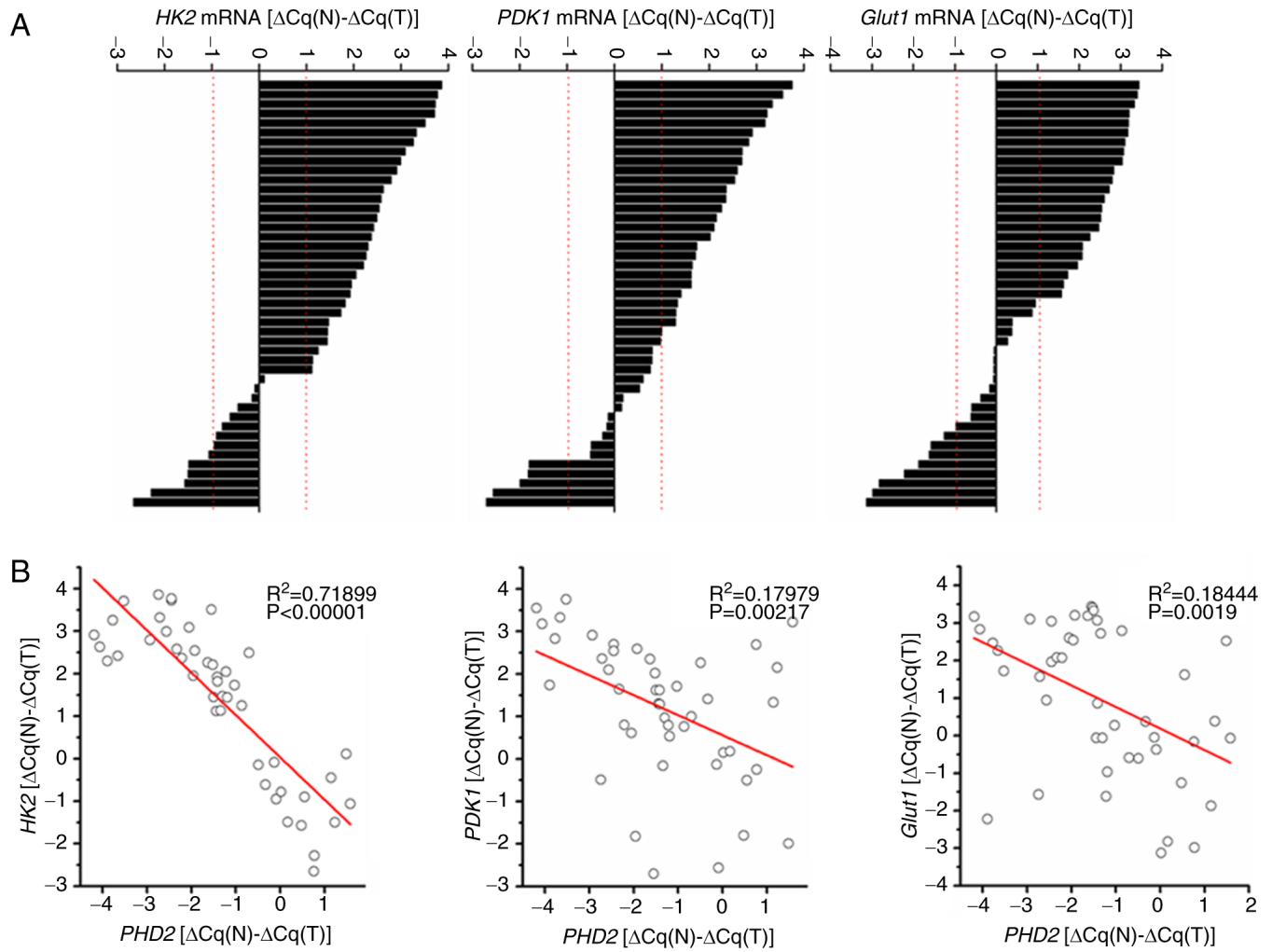

Figure 2. PHD2 is negatively correlated with glycolysis-related genes in human colorectal cancers. (A) Results of RT-qPCR detecting the expression of *HK2*, *PDK1*, or *GLUT1*. Δ*Cq(N)* and Δ*Cq(T)* indicate the Cq value of GAPDH was subtracted from Cq value of *HK2*, *PDK1*, or *GLUT1* in normal tissue or tumor tissue, respectively. Bar value: (Δ*Cq(N)*-Δ*Cq(T)*) depicts the fold-changes of *HK2*, *PDK1*, or *GLUT1* mRNA in tumor tissues vs. normal tissues and the difference is expressed in log2 scale. (B) Significant negative correlation between the relative mRNA expression level of *PHD2* (x-axis) and *HK2*, *PDK1*, or *GLUT1* (y-axis) in the colorectal cancer samples. PHD2, prolyl hydroxylase domain protein 2; RT-qPCR, reverse transcription-quantitative PCR.

Inhibition of PHD2 stimulates glycolytic activity in colorectal cancer cells. To confirm the role of PHD2 in tumor-associated glycolysis, *in vitro* glycolytic activity was examined in different human colorectal cancer cells with altered PHD2 expression levels. Among the tested colon cancer cell lines, SW480 cells presented the lowest, while Ls174t cells expressed the highest PHD2 protein expression (Fig. 1C). Therefore, PHD2 was overexpressed in SW480 cells (PHD2-OE) and PHD2 silenced in Ls174t cells (PHD2-KD) via lentiviral-based modification, respectively. Transduction efficiency and PHD2 expression were evaluated by immunoblotting analysis (Fig. 3A). As the data indicated a negative correlations between PHD2 and GLUT1, HK2 and PDK1, the protein level of these proteins was assessed in PHD2-OE cells and control cells (Fig. 3B). Compared with control counterparts, PHD2 overexpression significantly suppressed the expression of GLUT1, HK2 and PDK1 proteins.

Notably, PHD2 can also be inhibited *in vitro* by the competitive inhibitor dimethyloxalylglycine (DMOG), a cell-permeable 2-oxoglutarate analog (27). It was observed that 24 h exposure to DMOD led to a dose-dependent PHD2 reduction in colon cancer cells (0, 0.125, 0.25, 0.50, or 1.00 mM DMOG; Fig. 3C). Then, the role of PHD2 in glycolytic activity was further investigated by evaluating glucose uptake, lactate production and intracellular ATP/ADP ratio. It was found the glycolytic activity increased greatly in PHD2-KD cells and DMOG (0.5 mM) treated cells compared with the control group (Fig. 3D-F). By contrast, opposite effects could be observed in PHD2-OE cells (Fig. 3G-I). In addition, not only was a dose-dependent inhibitory effect of DMOG on PHD2 expression discovered but it was also noted that DMOG promoted glucose uptake, lactate production and intracellular ATP/ADP ratio in colon cancer cells in the same manner (Fig. 3J and K). Taken together, the results suggested that PHD2 may be involved in regulating glycolysis in colon cancer cells.

Regulation of glycolysis by PHD2 is independent of HIF-1α. As HIF-1α is the functionally characterized target of PHD2 (28), it was next determined whether the effects of PHD2 on glycolysis were dependent on HIF-1α. For this purpose, the present study generated HIF-1α deficient and HIF-1α/PHD2 double

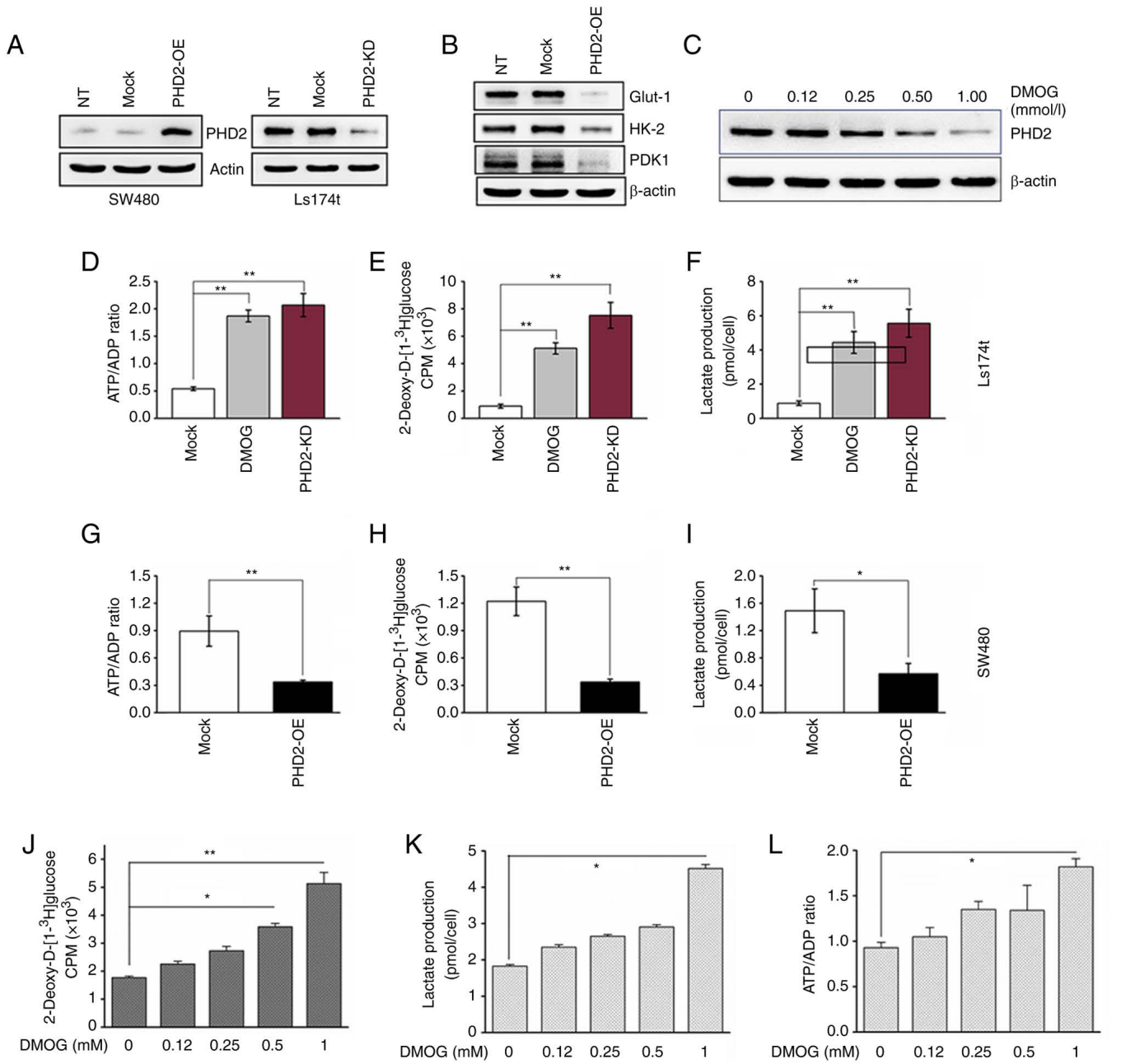

Figure 3. Inhibition of PHD2 promotes glycolysis in colorectal cancer cells. (A) PHD2 overexpression and silencing were confirmed by western blot analysis. (B) Expression of GLUT1, HK2 and PDK1 in SW480 cells with altered PHD2 expression. (C) Expression of PHD2 in Ls174t treated with DMOG at indicated concentrations for 24 h. (D and G) Quantitative analysis of the ATP/ADP ratio, (E and H) glucose uptake and (F and I) extracellular lactate in control, (0.5 mM) DMOG treated and PHD2-KD colon cancer cells. (J) Glucose uptake, (K) lactate production and (L) the ratio of ATP/ADP, were quantitatively analyzed in Ls174t cells preincubated in the medium containing 0-1 mM DMOG for 24 h (n=3; *P<0.05 and **P<0.01 vs. Mock). PHD2, prolyl hydroxylase domain protein 2; GLUT1, glucose transporter 1; HK2, hexokinease-2; PDK1, PHD kinase 1; DMOG, dimethyloxalylglycine; ATP, adenosine-5'-triphosphate; ADP, adenosine diphosphate; NT, non-transduced; Mock, non-targeting shRNA control (for PHD2 knockdown) or vehicle control (for PHD2 overexpression); PHD2-OE, PHD2 overexpression; PHD2-KD, PHD2-knockdown.

deficient colon cancer cells and the expression of HIF-1α and PHD2 was evaluated. HIF-1α expression was significantly suppressed by lentivirus-mediated silencing and PHD2 depletion elevated the expression of HIF-1α (Fig. 4A). In addition, it was noticed that cells with HIF-1α silenced did not promote glycolysis activity as reflected by no significant change in glucose uptake, lactate production and intracellular ATP/ADP ratio compared with parental cells (Fig. 4B-D). It also found that further knockdown of HIF-1α in colon cancer cells did not affect the PHD2 depletion-mediated promotive effects on colon cancer glycolysis activity (Fig. 4B-D). These findings prompted the hypothesis that PHD2-promoted glycolysis is independent of HIF-1α in colon cancer cells.

PHD2 inhibits glycolytic activity through IKKβ/NF-κB. Accumulating evidence has revealed that PHD2 has novel targets other than HIF-1α (29). NF-κB signaling components are notable candidate targets (30,31). Therefore, the present study determined how PHD2 affects NF-κB signaling activity and found that depletion of PHD2 led to upregulated nuclear p65

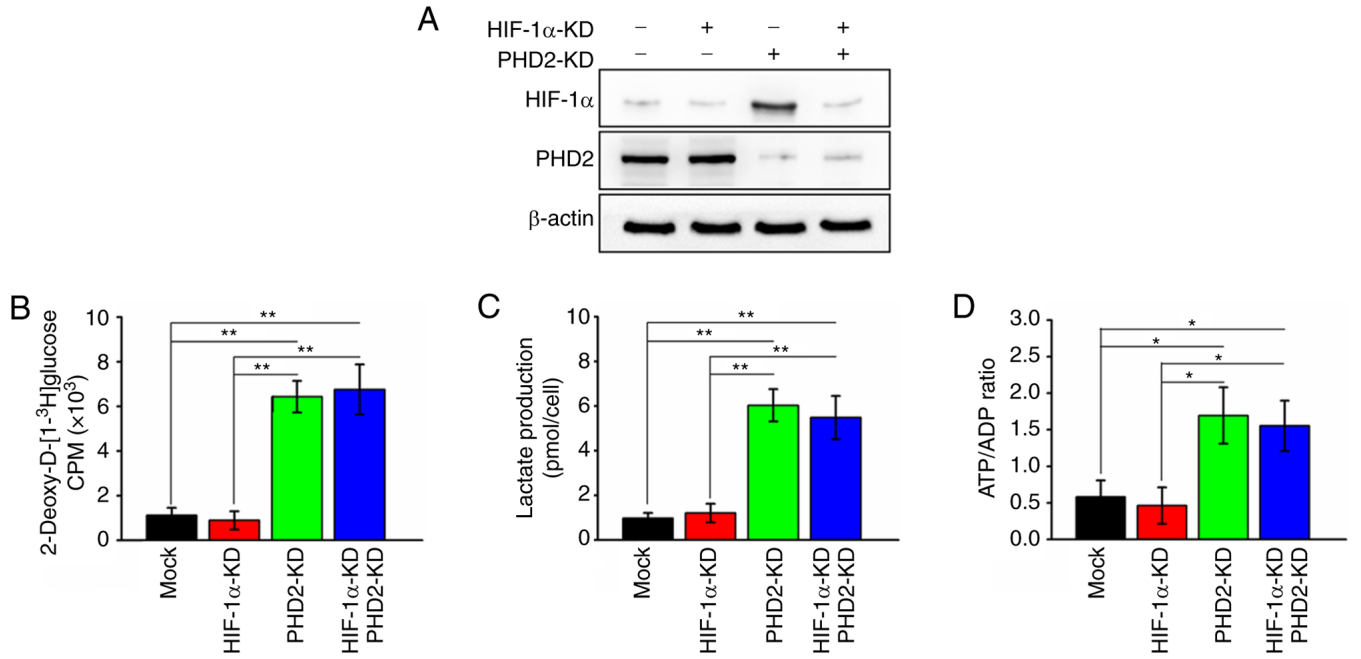

Figure 4. Regulation of glycolysis by PHD2 is HIF-1α-independent. (A) Representative results of western blot analysis detecting the expression of PHD2 and HIF-1α in Ls174t cells under indicated conditions. The ratio of (B) glucose uptake, (C) lactate production and (D) ATP/ADP ratio were quantitatively analyzed in control, HIF-1α silenced, PHD2 silenced and HIF-1α and PHD2 dual-silenced Ls174t cells (n=3; *P<0.05 and **P<0.01 vs. Mock or vs. HIF-1α-KD). PHD2, prolyl hydroxylase domain protein 2; HIF-1α hypoxia-inducible factor-1α; Mock, non-targeting shRNA control; PHD2-KD, PHD2 knockdown; HIF-1α-KD, HIF-1α knockdown.

subunit (Fig. 5A). Also, DMOG-mediated PHD2 suppression increased nuclear translocation of the p50 subunit (Fig. 5B). Notably, employing the IKKβ inhibitor (PDTC) or preventing the proteasomal degradation of IκB significantly attenuated the promotive effects of PHD2 knockdown (Fig. 5C-E) or PHD2 inhibition (Fig. 5F-H) on glycolysis in colon cancer cells. These findings indicated that PHD2 inhibits glycolytic activity through the IKKβ/NF-κB pathway.

PHD2 regulates the DNA binding properties of NF-κB. Prior studies have shown that NF-κB relies on IκB degradation to enter the nucleus and initiate transcription (32,33). In addition, NF-κB-dependent IκB synthesis is critical for NF-κB signaling termination (34). Furthermore, IKKβ has been reported to be the target of PHD2 (30,31). Thus, PHD2 may modulate NF-κB signaling in an IKKβ-dependent manner. Through immunoprecipitation assay, it was noted that, among the IKK isoforms, PHD2 preferably interacts with IKKβ (Fig. 6A). The present study further confirmed the interaction between PHD2 and IKKβ in cells with altered PHD2 expression. As shown, silencing or inhibiting PHD2 attenuated the PHD2-IKKβ interaction, while enhanced binding between PHD2 and IKKβ was observed in PHD2-OE cells (Fig. 6B and C). Meanwhile, the expression of p65 in the cytosol and nucleus was influenced by PHD2 overexpression (Fig. 6D), suggesting that PHD2 exerts its effect dose-dependently on IKKβ, presumably after p65 nuclear translocation.

PHD2 inhibits colorectal tumor growth in vivo and prolongs animal survival. To further characterize the functional significance of PHD2 on tumor growth *in vivo*, a subcutaneous xenograft model of colorectal cancer was established in SCID mice. Parental colon cancer cells (SW480 cells or Ls174t) or parental cells with or without PHD2 expression modulation (PHD2-OE, PHD2-KD, or Mock control) were subcutaneously inoculated into SCID mice. The data showed that colon cancer cells expressing control vectors grow into sizable tumors and were comparable to non-transduced cells (Fig. 7A and B). In addition, mice transplanted with Ls174t cells, which express a higher level of PHD2, developed smaller tumors than those implanted with SW480 cells (Fig. 7A and B). Notably, the data also demonstrated that tumor growth was significantly inhibited by PHD2 overexpression (Fig. 7A) but was dramatically promoted by PHD2 silencing (Fig. 7B). The result suggests a suppressive effect of exogenous PHD2 on human colorectal cancer progression *in vivo*. Additionally, the findings further revealed that Ki-67-positive cell proportion, representing the tumor proliferation index, was significantly reduced in mice transplanted with PHD2 overexpressing cancer cells and significantly higher in mice inoculated with PHD2 silencing cancer cells compared with the corresponding control mice (Fig. 7C-F). Kaplan-Meier curves were used to determine survival in each mouse group. The log-rank test demonstrated that PHD2 overexpression significantly prolonged the survival of tumor-bearing mice compared with the wild-type or mock control groups (P<0.01) (Fig. 7G). Again, as shown in Fig. 7H, PHD2 deficiency reduced the survival of tumor-bearing mice (P<0.01). Taken together, the data indicated that PHD2 markedly delayed tumor growth and increases survival of mice in a model of colorectal cancer.

Discussion

A promising therapeutic target selective for cancer cells may focus on their dependence on aerobic glycolysis for energy

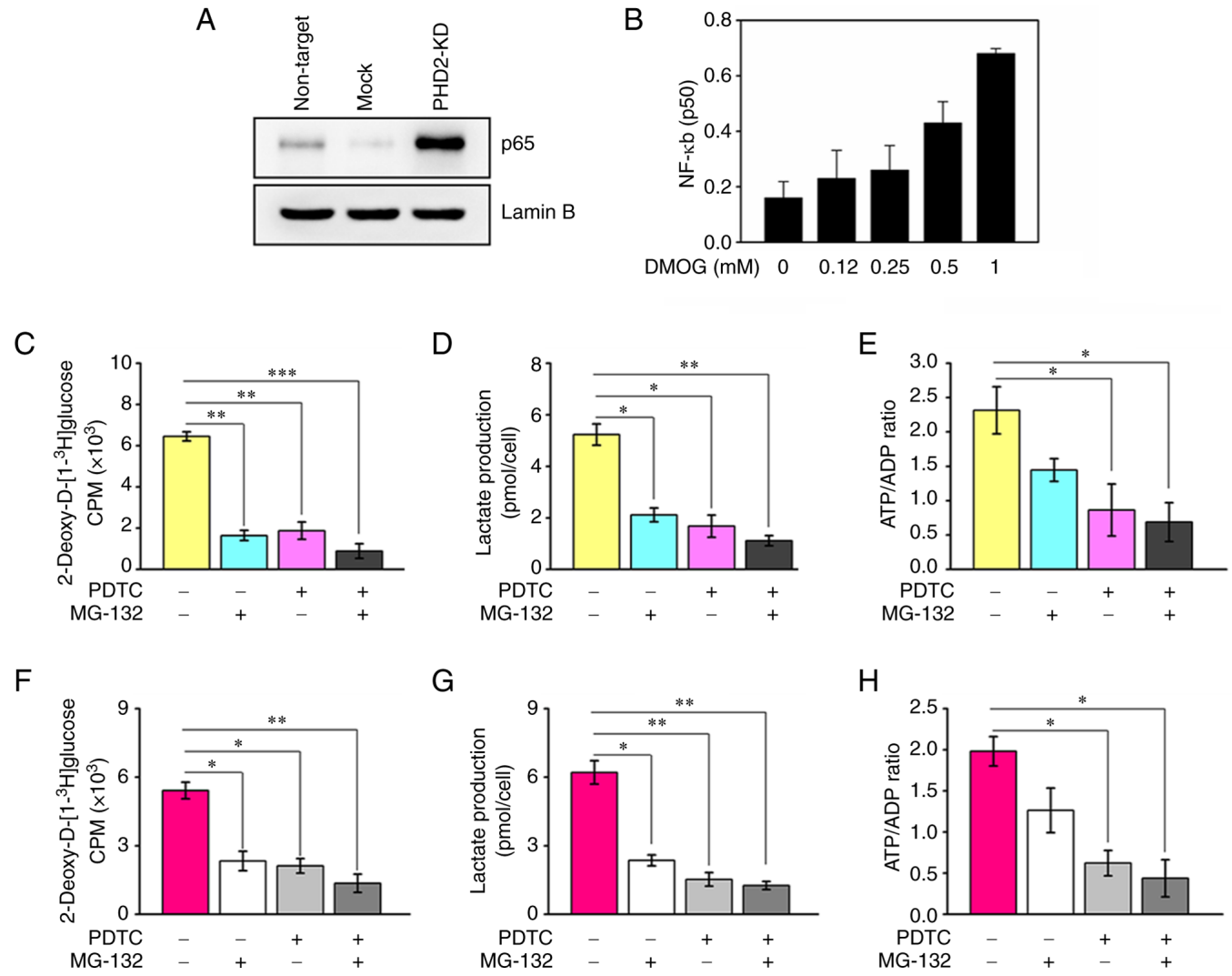

Figure 5. PHD2 inhibits glycolytic activity through IKKβ/NF-κB. (A) Nuclear p65 expression were determined by western blotting in Ls174t cells. (B) Expression of nuclear p50 was measured by ELISA assay. Quantified (C) glucose uptake (D) lactate production and (E) ATP/ADP ratio in PHD2-deficient Ls174t cells under indicated treatments (n=3; *P<0.05 and **P<0.01 vs. un-treated cells). Quantified (F) glucose uptake (G) lactate production and (H) ATP/ADP ratio in PHD2 inhibited Ls174t cells under indicated conditions (n=3; *P<0.05 and **P<0.01 vs. untreated cells). PHD2, prolyl hydroxylase domain protein 2; ATP, adenosine-5'-triphosphate; ADP, adenosine diphosphate; NT, non-transduced; Mock, non-targeting shRNA control; PHD2-KD, PHD2 knockdown.

metabolism (35). An increasing body of evidence has revealed that elevated glycolysis can enhance the *O*-glycosylation of IKKβ that subsequently triggers the activation of NF-κB signaling (36-38). Other studies have implicated IKKβ/NF-κB signaling in cancer cell metabolic stress adaption and energy metabolism, including colorectal cancer (39-42). In addition, PHD2-mediated activation of NF-κB signaling has been reported via the IκB-dependent pathway (31). However, whether PHD2 regulates glycolysis by modulating NF-κB signaling in colorectal cancer has not been well studied. The present study explored the crucial role of PHD2 in colon cancer cell glycolysis and investigated the potential regulatory effects of PHD2 in NF-κB signaling during glycolysis both *in vitro* and *in vivo*.

By interrogating PHD2 expression in human tissues and cell lines, it was found that PHD2 expression was significantly downregulated in cancerous tissues and cells, in agreement with previous findings (29,43). In association with PHD2 downregulation, the present study also observed elevated critical glycolytic enzymes, HK2, PDK1 and GLUT1, in human colorectal cancer tissues, negatively correlated with PHD2 expression level. The present *in vitro* study consistently reported that PHD2 overexpression decreased, while PHD2 depletion increased, the expression of GLUT1, HK2 and PDK1 in colon cancer cells. Similar findings have also been observed by other researchers (44). These findings highlight the importance of PHD2 in cancer glucose metabolism and shed light on the tumor-suppressive role of PHD2.

PHD2 has been reported to regulate HIF-1α proteasomal degradation via hydroxylation in normoxia (9). Meanwhile, in the presence of DMOG, or during hypoxia, PHD2 is suppressed. In turn, HIF-1α enters nuclear and mediates glycolysis (45). Similarly, upregulated HIF-1α in PHD2 depleted cells was observed. However, in contrast to the expectation that silencing HIF-1α in PHD2 deficient cells would affect the glucose metabolism, it was found that the PHD2-deficiency-enhanced glycolysis did not depend on the absence of HIF-1α. Notably, a previous study has demonstrated that depleting PHD2 in

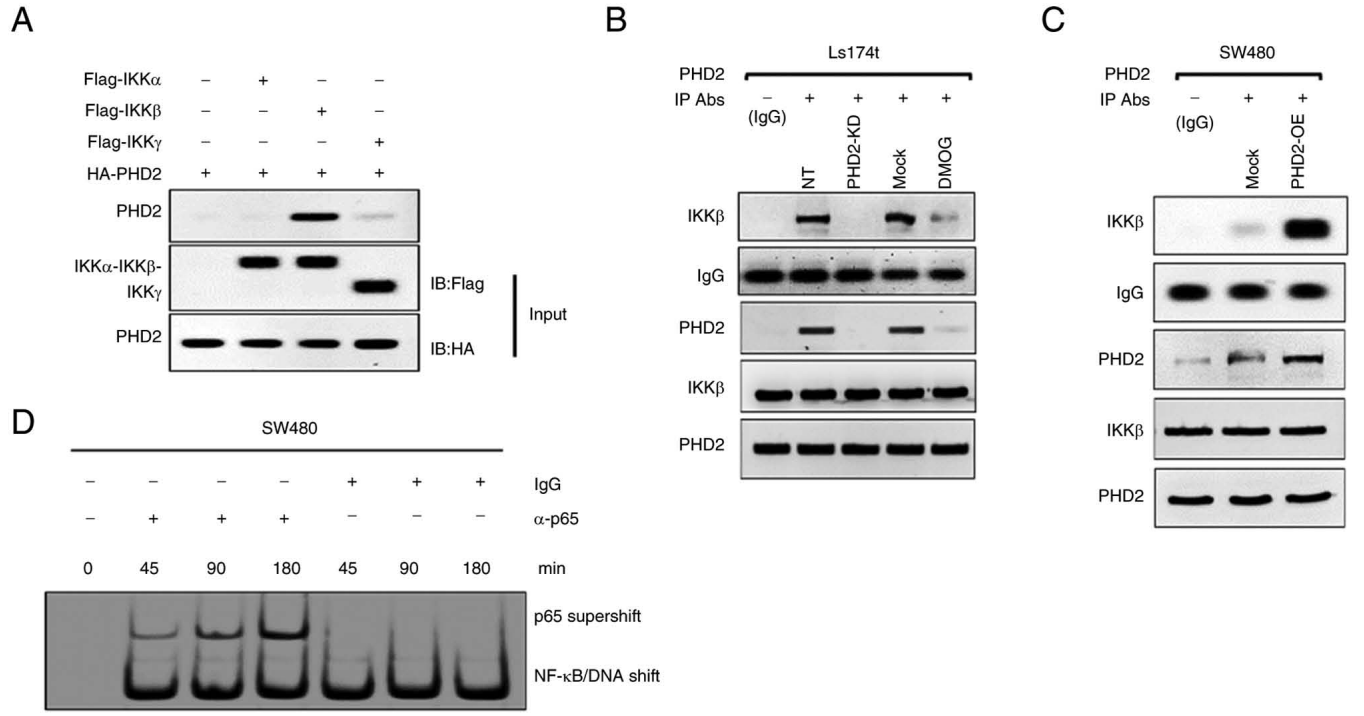

Figure 6. PHD2 regulates NF-κB DNA binding properties. (A) Results of immunoprecipitation evaluating the interactions between PHD2 and IKKα, IKKβ and IKKγ. Results of immunoprecipitation detecting the binding between IKKβ and PHD2 in colon cancer cells (B) with PHD2 depletion or suppression or (C) with PHD2 overexpression. (D) Representative results of supershift analysis of NF-kB complexes composed of p65/p50 heterodimers. PHD2, prolyl hydroxylase domain protein 2; NT, non-transduced; Mock, non-targeting shRNA control (for PHD2 knockdown) or vehicle control (for PHD2 overexpression); PHD2-OE, PHD2 overexpression; PHD2-KD, PHD2-knockdown.

colon cancer cells (HCT116) promotes tumor progression in a HIF-1α-independent but NF-κB-dependent manner (31). A recent study has shown that PHD2 activates NF-κB signaling independent of HIF-1 signaling (46). These studies, at least partially, support the findings of the present study. They also prompted the discovery of the 'talk' between PHD2 and NF-κB signaling. Notably, the results of the present study showed that PHD2 inhibitor DMOG induced NF-κB activation. These findings indicated that the HIF-hydroxylase activity in PHD2 is not necessary for NF-κB inhibition. A recent study revealed that PHD2 exerts an inhibitory effect on NF-κB signaling in colon cancer (47). In addition, PHD2 has been demonstrated to hydroxylate Ikkβ within a conserved motif (LXXLAP) in malignant cells from different organs, such as colon and cervix (30). Thus, targeting the PHD2/Ikkβ/NF-κB axis may be a promising therapeutic strategy for colorectal cancer.

To further validate the findings of the present study, an *in vivo* xenograft model was generated. In mice that received PHD2 overexpressing cells, a smaller tumor burden, extended survival and reduced tumor cell proliferation were observed. By contrast, mice receiving PHD2 deficient cells developed a larger tumor burden, shorter survival and enhanced cell proliferation. These data were consistent with findings in a recent study on colon cancer (47). Together, these data highlighted a tumor-suppressive role of PHD2 in colorectal cancer.

However, it has to be mentioned that a variety of molecules have been identified as PHD2 targets apart from Ikkβ and HIF-1α. For example, PHD2 has been reported to hydroxylate NDRG3 (48) and eukaryotic elongation factor 2 kinase (49), linked to tumor progression (50). Therefore, PHD2 may inhibit tumor progression through different pathways synergistically and these molecules and other un-identified participants may be involved in this process. However, due to the limited resources, the current study only focused on dissecting the role of the PHD2/Ikkβ/NF-κB axis during the glycolysis of colon cancer. The results would be more comprehensive if the crosstalk among these potential candidates could be explored simultaneously.

PHD3 competes with HSP90 for IKKβ binding, explaining why PHD modulation does not require its hydroxylase activity (51). PHDs share a conserved C-terminal domain with hydroxylase activity. It was hypothesized that PHD2 may modulate NF-κB signaling by means of IκκB hydroxylation.

NF-κB activation in colon cancer cells has been well documented in previous studies (52,53). The results from the current study revealed a decreased PHD2 in colorectal cancer. The present study also demonstrated that PHD2 terminated NF-κB signaling in colorectal cancer cells. Together, these findings shed light on the potential significance of PHD2 during colorectal cancer progression. In addition, the crosstalk between NF-κB and IKK connected cancer progression and glycolysis. The present study revealed that PHD2 depletion in colorectal cancer cells enhanced glycolysis (Fig. 3). These findings indicated a suppressive effect of PHD2 on colorectal cancer cells, at least to a certain degree, via the IKK/NF-κB axis. Furthermore, as aforementioned, PHD2 targets multiple proteins; it is possible that other proteins downstream of PHD2 also participate in this process. In addition to the associations between PHD2 expression level and colorectal tumor stage and overall survival of patients (23), the present study indicated the suppressive function of PHD2 in colorectal cancer.

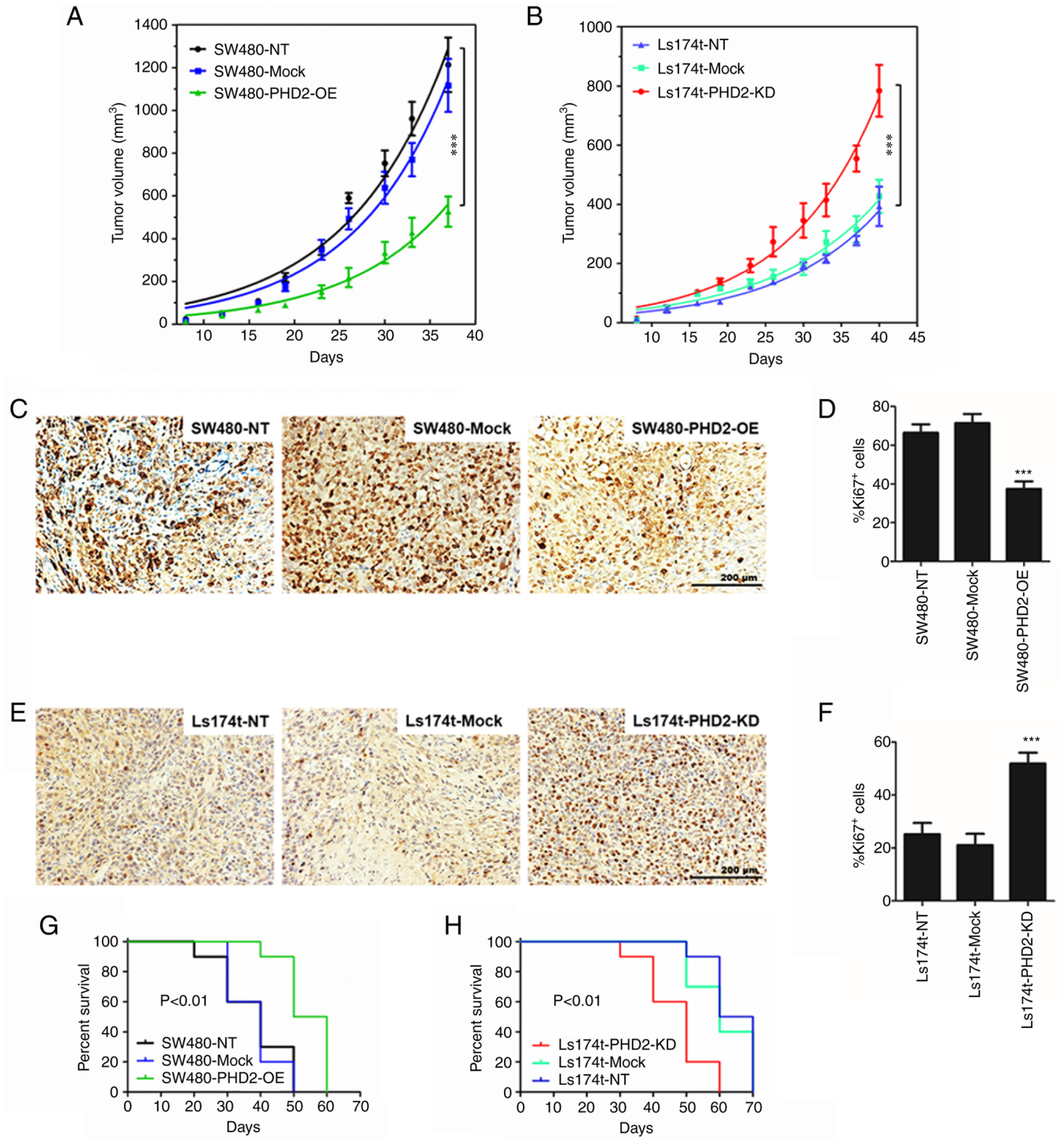

Figure 7. Evaluating the role of PHD2 in tumor progression *in vivo*. (A and B) Results of tumor volumes observed in mice transplanted with colon cancer cells with indicated PHD2 modulations. (n=6; ***P<0.001 vs. NT or Mock). (C-F) Representative images of immunohistochemistry analysis and quantitative results demonstrating the Ki67 expression in xenografts harvested from indicated groups. Scale bar, 200 μm. (n=6; ***P<0.001 vs. control groups). Kaplan-Meier survival curves were calculated for (G) SW480 subclones and (H) Ls174t subclones formed tumors as aforementioned (n=10; P<0.01 vs. control groups). PHD2, prolyl hydroxylase domain protein 2; NT, non-transduced; Mock, non-targeting shRNA control (for PHD2 knockdown) or vehicle control (for PHD2 overexpression).

NF-κB signaling-induced cancer cell survival and therapy resistance have been reported in numerous malignancies, including colorectal cancer. Elevated NF-κB activity has been observed in colorectal cancer cells treated with chemotherapeutics (54); therefore, targeting NF-κB is expected to potentiate conventional therapeutic regimens in comprehensive strategies. Furthermore, an increasing number of studies indicate that metabolic pathways may be favorable targets (55,56). For example, the present study found that PHD2 inhibited NF-κB and knockdown of PHD2 led to upregulation of glycolysis in colorectal cancer cells, which essentially increases tumor cell proliferation and reduces animal survival. Taken together, evaluating PHD2 level may facilitate the selection of a targeted therapeutic regime for colorectal cancer with elevated NF-κB activity.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GX and LX designed the study and wrote the manuscript. HW, LX, WY, SW and GX performed the experiments. LX, HW and SW analyzed the data. LX and GX advised on experimental procedures and revision of the paper. GX and LX confirm the authenticity of all the raw data. All authors contributed to this manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the 1964 Declaration of Helsinki. The protocol of immunohistochemistry for patient tissues was approved by the Ethics Committee of the First Affiliated Hospital (Southwest Hospital), the Third Military Medical University [Army Medical University; approval no. 2012 (12)] and all patients or family members involved provided written informed consent. Animal studies were performed under a protocol approved by the Army Medical University Institutional Animal Care and Use Committee (approval no. AMUWEC20232707).

Patient consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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