



Prolyl hydroxylase 3 involvement in lung cancer progression under hypoxic conditions: association with hypoxia-inducible factor-1 α and pyruvate kinase M2

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Background: Previous studies have suggested that the functions of prolyl hydroxylase 3 (PHD3) in tumor growth, apoptosis and angiogenesis are essentially dependent on hypoxia-inducible factor (HIF)-1 α signaling. Nevertheless, whether PHD3 represents a promising tumor suppressor target remains to be clarified. To provide insight into the therapeutic potential of PHD3 in lung cancer, this study examined the effects of PHD3 expression on HIF-1 α and pyruvate kinase M2 (PKM2), as well as on lung cancer cell proliferation, migration, and invasion.

Methods: The model of hypoxia was established in A549 and SK-MES-1 cells with 200 μ M CoCl₂ treatment, and verified by western blot and immunocytochemical staining. The expression levels of PKM2 and HIF-1 α were determined by western blot after overexpression or depletion of PHD3 in A549 and SK-MES-1 cells. In addition, cell viability, migration and invasion were measured, respectively.

Results: Establishment of hypoxia in A549 and SK-MES-1 cells resulted in significant decreases in PHD3 expression and remarkable increase in PKM2 expression in 24 hrs. Overexpression of PHD3 in A549 and SK-MES-1 cells decreased HIF-1 α and PKM2 expression. In contrast, PHD3 knockdown increased HIF-1 α and PKM2 ($P < 0.05$). In addition, the viability, migration and invasion of A549 and SK-MES-1 cells were significantly decreased with PHD3 overexpression, but dramatically increased with PHD3 depletion ($P < 0.05$).

Conclusions: PHD3 is involved in lung cancer progression, and might be a promising therapeutic target for cancers.

Keywords: Prolyl hydroxylase 3 (PHD3); lung cancer; hypoxia-inducible factor-1 α (HIF-1 α); pyruvate kinase M2 (PKM2)

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Introduction

Microenvironmental hypoxia, a classic feature of many human malignancies, plays an important role in the growth and progression of tumors, promoting angiogenesis, metastasis, and resistance to therapy (1). Hypoxia-inducible

factors (HIFs) are master regulators of oxygen homeostasis in response to hypoxia. HIF-1, a heterodimeric complex protein is composed of a constitutively expressed nuclear subunit HIF-1 β and an oxygen dependent subunit (HIF-1 α) both of which are ubiquitously expressed in human tissues (2). HIF-1 mediates the transcription of genes involved in

oxygen delivery and metabolic adaptation to hypoxia, and plays essential roles in tumor proliferation, metabolism, angiogenesis, metastasis and differentiation (3). The expression of HIF-1 α correlates with poor patient prognosis in a variety of cancers (4-6). Evidence demonstrates that down-regulation of HIF-1 α inhibits the proliferation and invasion of lung cancer cells (7). Currently, HIF-1 α inhibitors acting through several molecular mechanisms are being investigated for therapeutic purposes (8).

Three HIF prolyl hydroxylase (PHD1, PHD2 and PHD3) which are oxygen sensors regulate the stability of HIFs by post-translational hydroxylation of proline residues in its α subunit in an oxygen-dependent manner, promoting the ubiquitination of HIF- α (9). The prolyl hydroxylase 3 (PHD3), which is regulated by hypoxia, could play important roles in tumor progression by controlling epidermal growth factor receptor (EGFR) activity (10) and hypoxic vascular endothelial growth factor secretion within the hypoxic microenvironment of the tumor (11). Pyruvate kinase M2 (PKM2) has emerging roles in cancer metabolism, growth and progression (12). Miao *et al.* demonstrated that down-regulation of PKM2 could inhibit cell proliferation, suppress cell migration and invasion, and induce apoptosis (13). Luo *et al.* reported that PHD3 mediates the binding of PKM2 to HIF-1 α , and regulates PKM2 function (14). In addition, HIF-1 activates the transcription of PKM2 and PHD3.

Previous studies have suggested that the functions of PHD3 in tumor growth, apoptosis and angiogenesis are essentially dependent on HIF-1 signaling (11). It is also reported that expression of PHDs and of the asparagine hydroxylase FIH relates to an activated HIF pathway in non-small cell lung cancer (15). Moreover, Tennant *et al.* found that PHD3 was required to induce apoptosis and inhibit tumor growth *in vivo*, indicating that PHD3 could be a valid target for anti-cancer therapies (16). Nevertheless, whether PHD3 represents a promising tumor suppressor target remains to be clarified. To provide insight into the therapeutic potential of PHD3 in cancers, this study examined the effects of PHD3 dysregulation on HIF-1 α and PKM2 expression, as well as on the lung cell proliferation, migration, and invasion.

Methods

Cell culture and induction of hypoxia

A non-small-cell lung cancer cell line A549 and a lung

squamous cell carcinoma cell line SK-MES-1 were purchased from American Type Culture Collection (Manassas, VA, USA). They were cultured in DMEM (12430-054, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100,000 U/L penicillin and 100 mg/L streptomycin (15140, Gibco) for 48 h at 37 °C in a 5% CO₂ atmosphere. A549 (2 \times 10⁵ cells per well) and SK-MES-1 (2 \times 10⁶ cells per well) cells were seeded onto six-well plates and incubated overnight. Cells were then treated with 200 μ M cobalt chloride (CoCl₂, Sinopharm Chemical, USA) for 12 or 24 h.

Detection of HIF-1 α , PHD3, and PKM2 expression

For detection of HIF-1 α , PHD3, and PKM2 protein expression, western blots were performed as previously described by Acker *et al.* (17), hypoxia-treated A549 and SK-MES-1 cells were lysed in RIPA buffer (Beyotime, Jiangsu, China), and lysate protein concentration was measured with a BCA Protein Assay Kit (P0010S, Beyotime). Western blots were conducted using the following antibodies: HIF-1 α (1:1,000, 20960-1-AP, Proteintech), PKM2 (1:1,000, 15822-1-AP, Proteintech), and PHD3 (1:1,000, 18325-1-AP, Proteintech). GAPDH (10494-1-AP, Proteintech, 1:1,000) was used as a loading control. Secondary antibodies were conjugated to horseradish peroxidase (1:10,000, Jackson ImmunoResearch, West Grove, PA, USA) and protein signals were detected by enhanced chemiluminescence.

Immunocytochemical staining was carried out as in Comati *et al.* (18) using the antibodies listed above. Antigen-antibody complexes were visualized with diaminobenzidine solution (Beijing Zhongshan Jinqiao Biological Technology Co., Beijing, China) and nuclei were labeled with hematoxylin (517-28-2, Solarbio, China).

Plasmids and cell transfection

DNA encoding the human *PHD3* gene was synthesized by Sangon Biotech (Shanghai, China), inserted into pUC57, and transformed into *E. coli* One Shot Top10 competent cells. The recombinant plasmids were extracted using standard methods and verified by sequencing. The target gene was then digested from the pUC57 vector and ligated into the pcDNA3.1 expression vector to construct overexpression vectors, named pcDNA3.1-PHD3.

A549 and SK-MES-1 cells grown in six-well plates were transfected with pcDNA3.1-PHD3 or small interfering RNA (siRNA) against PHD3 (PHD3 siRNA,

100 nM) (diluted with 250 μ L OPTI-MEM, Tuoran biotend biotechnology Co., Ltd, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific Inc., Shanghai, China) for 48 h. The expression of PHD3 in A549 and SK-MES-1 cells after overexpression and knockdown was verified by western blot.

Cell viability analysis

The A549 and SK-MES-1 cells were incubated with 10 μ L CCK-8 (CK04, Dojindo Corp., Japan) for 1 h at 37 $^{\circ}$ C. The optical density (OD) at 450 nm was measured using an Infinite M1000 PRO plate reader (TECAN, Morrisville, NC, USA).

Transwell and Matrigel invasion assays

The A549 and SK-MES-1 cells were seeded into the upper chamber of Transwell plates (Corning Incorporated, Corning, NY, USA), and 600 μ L DMEM containing 10% FBS was added to the lower chamber. After 24 h incubation, cells in the bottom chamber were fixed with 4% buffered neutral formalin for 10 min, stained with 0.1% crystal violet, and examined by electron microscopy (200 \times). For the Matrigel invasion assay, the experiment was conducted in Matrigel Matrix-coated Transwell chambers (Corning).

Statistical analysis

All data were presented as mean \pm standard error (SEM). GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for data analysis. $P < 0.05$ indicated significant difference.

Results

Influence of hypoxia on PHD3 and PKM2 expression

We established a hypoxia model in A549 and SK-MES-1 cells, and examined the expression of HIF-1 α by western blot. As shown in *Figure 1*, the expression level of HIF-1 α was increased in both A549 and SK-MES-1 cells in a time-dependent manner ($P < 0.001$). We also examined the expression levels of PHD3 and PKM2 by western blot (*Figure 1A*) and immunocytochemical staining (*Figure 1B*). The expression of PHD3 in hypoxic A549 and SK-MES-1 cells decreased over time, while PKM2 expression increased ($P < 0.01$).

Overexpression or knockdown of PHD3

Since PHD3 expression decreased in hypoxic A549 and SK-MES-1 cells, we further investigated its role using overexpression and depletion experiments. As shown in *Figure 2A*, PHD3 expression was significantly increased in pcDNA3.1-PHD3-transfected A549 or SK-MES-1 cells compared to pcDNA3.1 control-transfected cells ($P < 0.001$).

Three siRNA sequences were used to silence PHD3 expression. As shown in *Figure 2B*, PHD3 protein expression was significantly reduced in PHD3 siRNA-transfected A549 or SK-MES-1 cells compared to the controls ($P < 0.05$). Among the three siRNAs, siRNA1 showed the highest silencing rate in both A549 and SK-MES-1 cells and was used for further experiments.

Effect of PHD3 expression changes on PKM2 and HIF-1 α

The effects of overexpression and knockdown of PHD3 on the expression of PKM2 and HIF1 α in A549 and SK-MES-1 cells were detected by western blot. The results showed that overexpression of PHD3 markedly decreased the expression of PKM2 and HIF-1 α in both A549 and SK-MES-1 cells (*Figure 3A*, $P < 0.001$). Conversely, knockdown of PHD3 increased PKM2 and HIF-1 α expression in the two cell lines (*Figure 3B*, $P < 0.01$).

Effect of PHD3 expression changes on lung cancer cells

The effects of PHD3 overexpression and knockdown on cell viability, migration and invasion of A549 and SK-MES-1 cells under hypoxic conditions were assessed, respectively. As shown in *Figure 4A,B,C*, the viability, migration, and invasion were significantly decreased in A549 cells overexpressing PHD3 under hypoxic conditions, compared to negative controls ($P < 0.05$). Conversely, the viability, migration, and invasion of A549 cells were significantly increased after PHD3 knockdown (*Figure 4D,E,F,G,H*, $P < 0.05$). In addition, compared to negative controls, the viability, migration, and invasion of SK-MES-1 cells were also significantly decreased after overexpression of PHD3 under hypoxic conditions (*Figure 5A,B,C,D,E*, $P < 0.001$), but remarkably increased after PHD3 knockdown (*Figure 5F,G,H,I,J*, $P < 0.05$).

Discussion

Hypoxia is associated with prognosis and tumor control

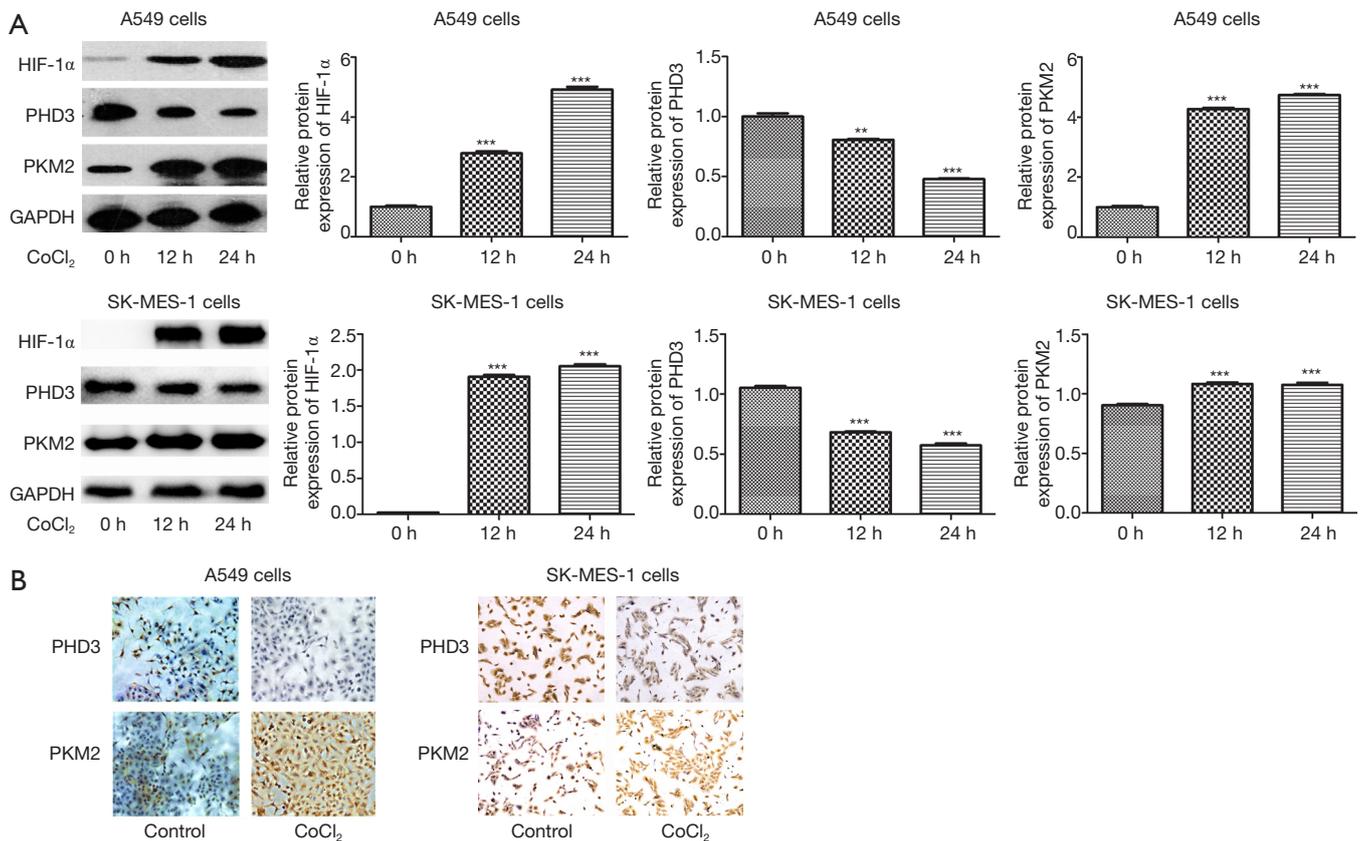


Figure 1 Expression of HIF-1α, PHD3, and PKM2 in hypoxic A549 and SK-MES-1 cells, detected by western blot (A) and immunohistochemistry (B). **P<0.01, and ***P<0.001. Magnification: 100×.

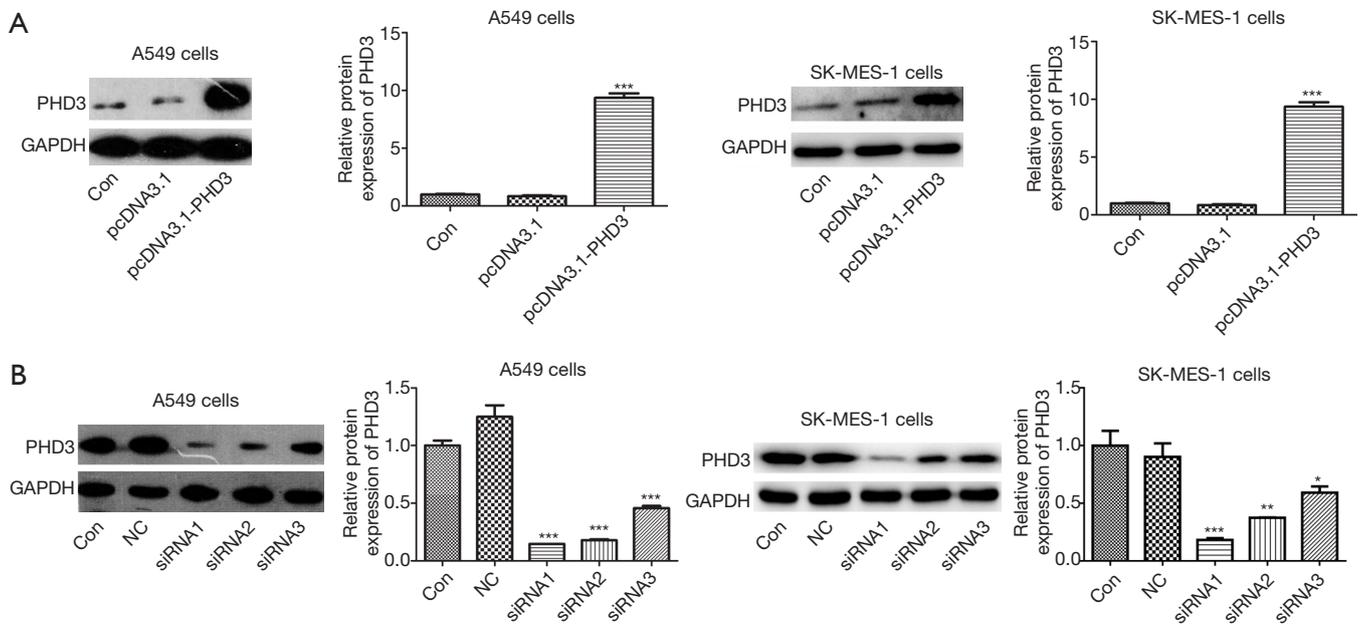


Figure 2 PHD3 protein levels in A549 and SK-MES-1 cells with overexpression (A) or knockdown (B) of PHD3. Con: control, NC: negative control. *P<0.05, **P<0.01, and ***P<0.001.

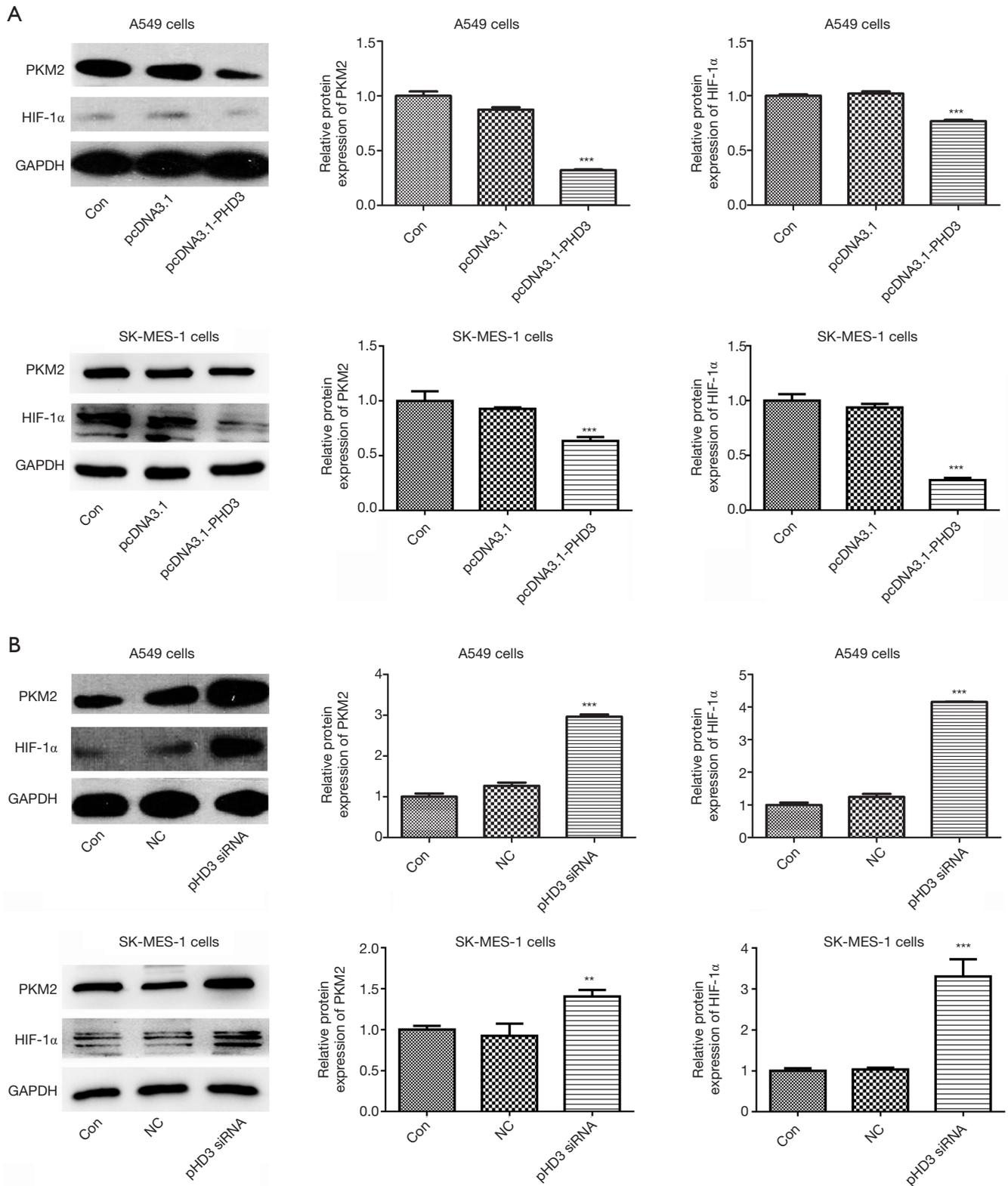


Figure 3 Expression of HIF-1α and PKM2 in A549 and SK-MES-1 cells with overexpression (A) or knockdown (B) of PHD3. Con: control, NC: negative control. **P<0.01, and ***P<0.001.

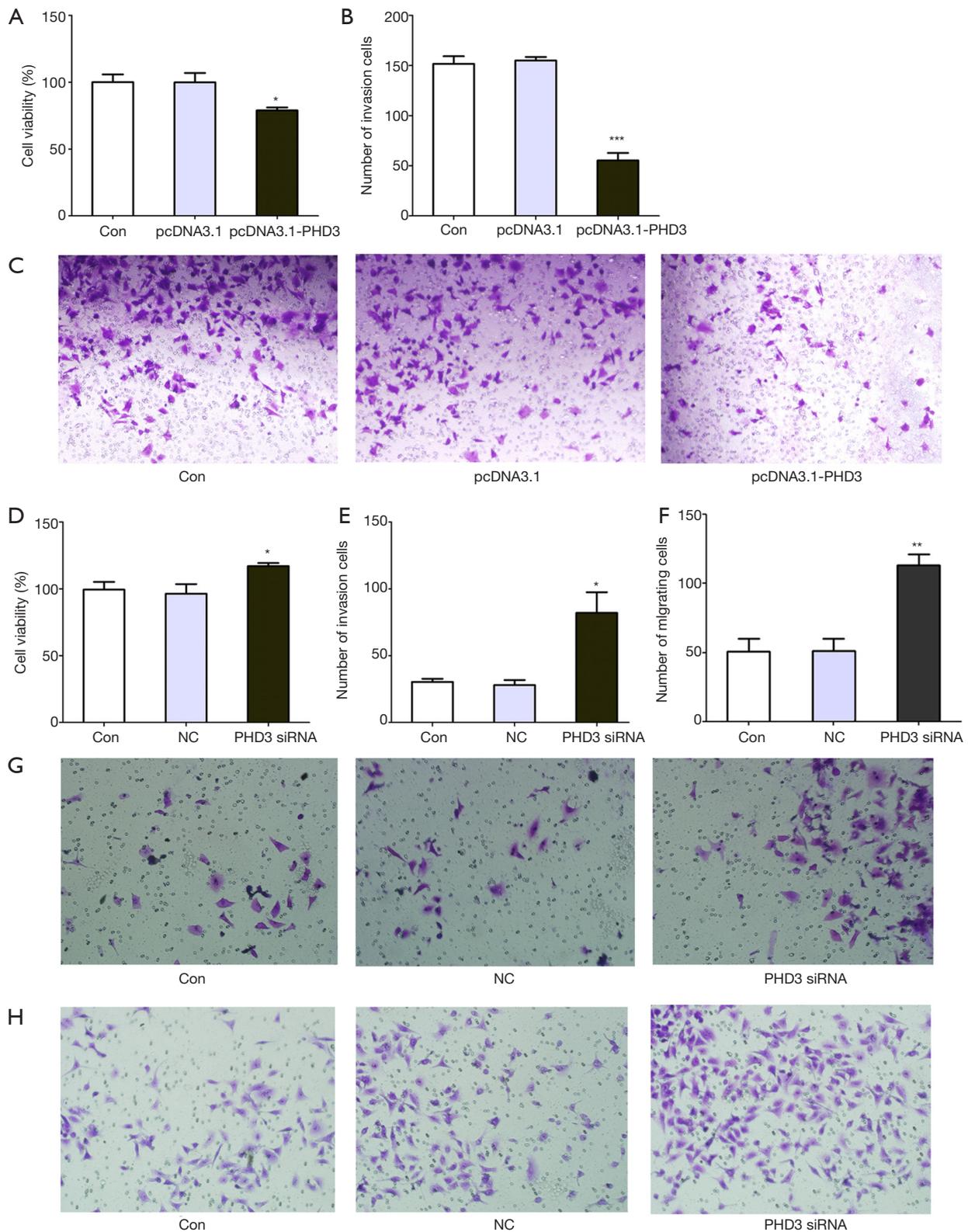


Figure 4 Cell viability (A) and invasion (B-C) were examined in PHD3-overexpressing A549 cells. Cell viability (D), invasion (E and G), and migration (F and H) were examined in PHD3-depleted A549 cells. Con: control, NC: negative control. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Magnification: 100 \times .

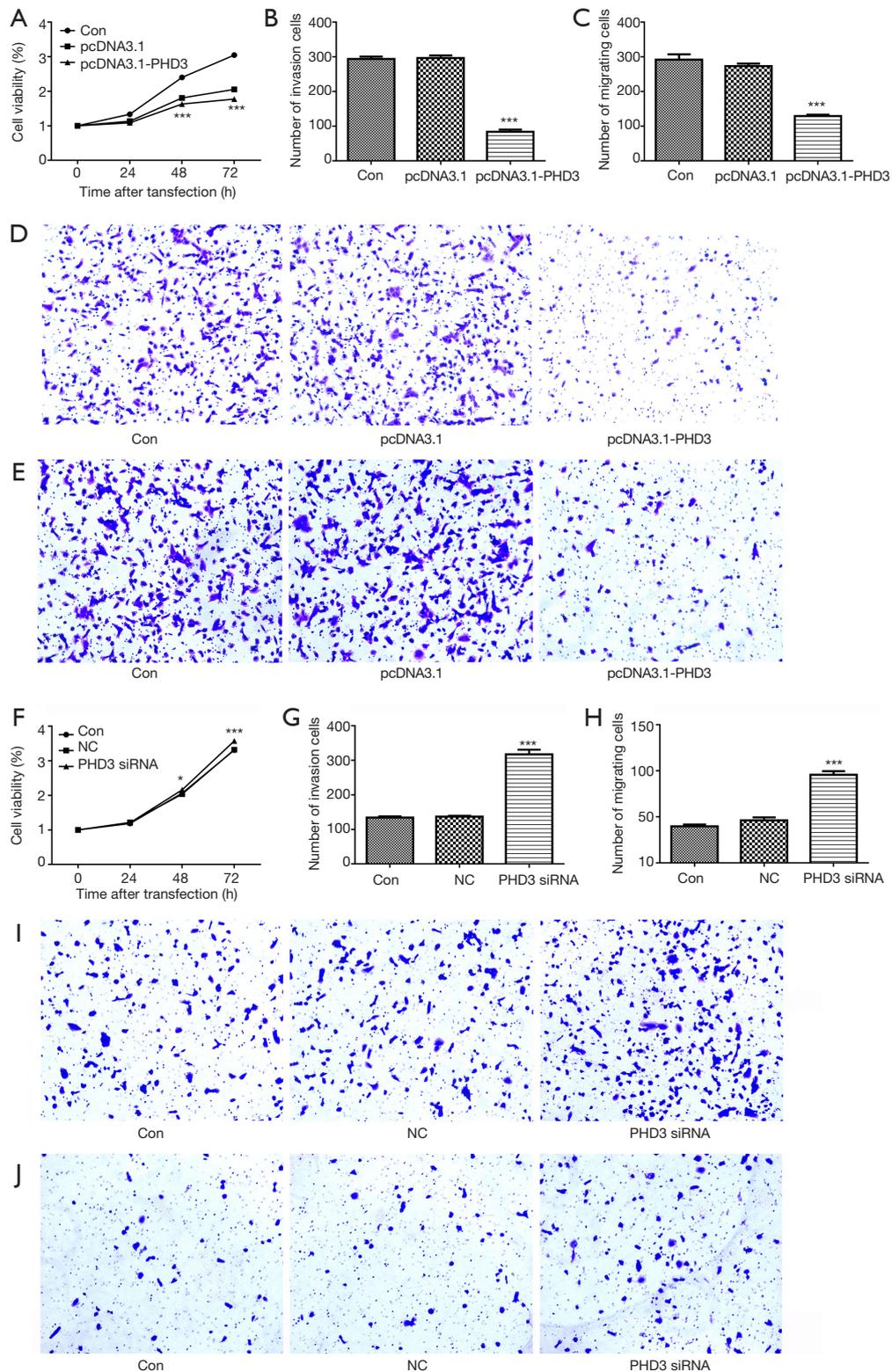


Figure 5 Cell viability (A), invasion (B and D), and migration (C and E) were examined in PHD3-overexpressing SK-MES-1 cells. Cell viability (F), invasion (G and I), and migration (H and J) were examined in PHD3-depleted SK-MES-1 cells. Con, control; NC, negative control. * $P < 0.05$, *** $P < 0.001$. Magnification: 100 \times .

in many cancers, including lung cancer (19). Moreover, PHD3 has been reported to play crucial role of PHD3 in many types of cancers. For instance, PHD3 is shown to regulate differentiation, tumor growth and angiogenesis in pancreatic cancer (11); overexpression of PHD3 can inhibit the migratory and invasive capacity of gastric cancer cells (20); and PHD3 inhibits lung cancer metastasis and restored sensitivity to erlotinib (21). However, the regulatory mechanism of PHD3 in lung cancer has not been clarified. This study investigated the effects of PHD3 expression on the expression of PKM2 and HIF-1 α , as well as cell biological characteristics of lung cancer cells, to examine the mechanism of action and therapeutic potential of PHD3 in lung cancer.

CoCl₂ is a hypoxia-mimicking agent, commonly used to induce hypoxia *in vitro* (22). After treatment with CoCl₂, the expression of PKM2 and HIF-1 α was significantly increased in A549 and SK-MES-1 cells, while PHD3 expression was relatively decreased. This was consistent with previous studies (23-25), indicating that the hypoxic model was constructed successfully. PHDs, which can hydroxylate key proline residues in HIF-1 α , are strikingly sensitive to graded levels of hypoxia *in vitro* (26). It is thought that CoCl₂ stabilizes HIF-1 α via inhibiting the activity of PHD enzymes (27).

To further explore the relationship between PHD3, HIF-1 α , and PKM2, we examined the effects of overexpression and knockdown of PHD3. Increased expression of PHD3 is induced by HIF-1 under hypoxic conditions (14). Interaction of PKM2 with PHD3 enhances PKM2 binding to HIF-1 α (14). PHDs inactivate HIFs in normoxia by activating degradation of the HIF- α subunit (28). In this study, PHD3 overexpression remarkably inhibited PKM2 and HIF-1 α expression, while knockdown of PHD3 significantly upregulated their expression, suggesting that expression of HIF-1 α and PKM2 were significantly negatively influenced by the expression level of PHD3. Thus, we hypothesize that a feedback loop involving HIF-1 α , PKM2, and PHD3 could participate in cancer progression.

The dimer form of PKM2 leads to anabolic metabolism of glucose for energy production, and contributes to the synthesis of DNA and lipids required for cell proliferation and tumor growth (12). Chu *et al.* demonstrated that apoptosis and autophagy are significantly induced in A549 cells by inhibiting PKM2 expression (29). Sun *et al.* reported that PKM2 could regulate cell proliferation and invasion by activating glycolysis and lipid synthesis in

lung adenocarcinoma (30), while Miao *et al.* reported that PKM2 could promote cell invasion in pancreatic ductal adenocarcinoma (13). PKM2 is also reported to control apoptosis in human leukemia cell lines (31). HIF-1 α accumulates due to decreased prolyl hydroxylase activity in hypoxia, potently enhances glycolytic metabolism, and interacts with c-Myc to control the cell cycle through the regulation of cyclins and cyclin-dependent kinase inhibitors (32). HIF-1 α also regulates migration in microglial cells (33). Additionally, it has been demonstrated that inhibition of HIF-1 α expression decreases proliferation and invasion of non-small cell lung cancer NCI-H157 cells (7) and enhances apoptosis (34). Increased PHD3 expression has been found to inhibit proliferation and enhance apoptosis through multiple mechanisms, including the regulation of EGFR (35) and HIF-1 signaling (36). Chen *et al.* demonstrated that enhanced PHD3 expression was negatively correlated with Bcl-2 expression, thus playing potential role in inducing apoptosis in non-small cell lung cancer (37). In line with the previous studies abovementioned, we found that PHD3 overexpression significantly decreased the viability and invasiveness of lung cancer cells with the inhibition of HIF-1 α and PKM2 expression.

However, this study had also several limitations. First, HIF-1 α target gene expression was not detected to further state the regulation of PHD3 on HIF-1 α -mediated biological processes. Second, CoCl₂ treatment was used to mimic hypoxic conditions. Future studies are needed to determine the results in the actual hypoxic condition.

Conclusions

Taken together, this work suggests that PHD3, PKM2, and HIF-1 α may have linked roles in the control of proliferation, migration and invasion of lung cancer cells. In this study, abnormal expression of PHD3 significantly influenced lung cancer cells; however, the precise mechanism remains to be elucidated. Additional investigations are still required to investigate the roles of PHD3 in tumor development and its potential as a target for anti-cancer therapies.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Ethical approval was obtained from the Fifth People's Hospital of Shanghai Fudan University Research Ethics Committee. Written informed consent was obtained for each participant, and the study was approved by the Hospital Ethics Committee.

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