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ORIGINAL ARTICLE

High Expression of PhospholipaseD2 Induced by Hypoxia Promotes Proliferation of Colon Cancer Cells through Activating NF- κ Bp65 Signaling Pathway

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Abstract
Hypoxia is a typical feature of colon cancer occurrence and progression. We have reported that high expression and activity of PhospholipaseD2 (PLD2) induced by hypoxia in colon cancer cells. In order to further investigate the role of PLD2 in colon cancer under hypoxic conditions. MTT assay was used to detect the proliferation of human colon cancer cells (SW480 and SW620) under hypoxic conditions by decrease the PLD2 gene expression or inhibit the activity of PLD2. Expression level of p-P65/T-P65 and Cyclin D1 were detected in those cells treated as above through using western blot and RT-PCR analysis. Effect of NF-κBp65 inhibitor (BAY-117082) on the proliferation and expression level of Cyclin D1 and PLD2 of colon cancer cells under hypoxic conditions were further analysised. As a result, decreased the expression of PLD2 or inhibited the activity of PLD2 leaded to the proliferation of hypoxia colon cancer cells reduced, and along with the expression level of p-P65/T-P65 and Cyclin D1 reduced. However, inhibition the expression level of p-P65/T-P65 lead to the proliferation and expression of Cyclin D1 in those hypoxia colon cancer cells also reduced. In vivo growth decreased in response to PLD2 and NF-κBp65 inhibition. Our study indicates that high expression of PLD2 induced by hypoxia promotes the proliferation of colon cancer cells, and it may elevate the expression level of Cyclin D1 through activating NF-κBp65 signaling pathway. Inhibition of the PLD2 expression may provide a new clue for treatment for colon cancer.

Keywords Colon cancer · PLD2 · Proliferation · NF-κBp65 · Hypoxia

Introduction

Colon cancer is one of the most common cancers in the world, and it is a leading cause of cancer-related death worldwide [1]. Although treatment strategies made a great progress in several passed decades, the survival rate of the patients with colon cancer remains very poor. In order to change this serve situation, further study the mechanism of colon cancer occurrence and progression is urgent. Since carcinogenesis is a multistage process, involving oncogene activation and tumor suppressor inactivation [2] and many factors have been considered that play important roles in this process [3]. In view of this topic, more and more researchers are interested in looking for new factors that associate with cancer occurrence and progression.

PLD2 as a member of phospholipaseD(PLD) family, which has been attracted widely interest due to its roles in the development and progression of many malignant tumors. So far, gastric carcinoma, lung cancer, gioma, kidney and breast cancer [4–8] have been reported to be associated with PLD2. In colon cancer, the expression and activity of PLD2 were significantly elevated [9, 10], and polymorphism of PLD2 gene has association with the prevalence of colorectal cancer [11]. Our research group also found that the expression of PLD2 elevated in colon cancer tissues and cells, and hypoxia induce high expression and activity of PLD2 in colon cancer cells

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[12, 13]. But the concrete mechanism of PLD2 involved in the development and progression of colon cancer was needed to be further illuminated.

In this study, we aim to detect the effect of PLD2 on the proliferation of hypoxia colon cancer cells and possible mechanism. The results showed that decreased PLD2 expression by siRNA or inhibition PLD2 activity could reduce the proliferation of colon cancer cells under hypoxic conditions. Moreover, PLD2 regulates the proliferation of colon cancer cells may up-regulates the expression of Cyclin D1 through activating NF- κ Bp65 signaling pathway. Those results may provide a new clue for the treatment target for colon cancer.

Results

PLD2 Promotes the Proliferation of Colon Cancer Cells under Hypoxic Conditions

SW620 and SW480 cells were divided into normoxia group, hypoxia group, control group, and PLD2 interference group. Normoxia group cells were cultured in normoxia conditions, and all cells of other three groups were cultured in hypoxia microenvironment for 24 h. Then the mRNA and protein expression level of PLD2 in those cells were detected by RT-PCR and western blot, respectively. The proliferation of those cells was detected by MTT assay. As a result, the siRNA targeting PLD2 significantly reduced the mRNA ($p < 0{,}01$, Fig. 1a) and protein ($p < 0.01$, Fig. 1b) level of PLD2 in hypoxia colon cancer cells. As shown in Fig. 1c, hypoxia induce the proliferation of colon cancer cells reduced compare with normoxia group ($p < 0.05$),while the proliferation of cells in PLD2 interference group was further reduced compare to the control group ($P < 0.05$), but there is no difference between hypoxia and control group ($P > 0.05$).

In order to examine the impact of PLD2 activity inhibitor (VU0364739) on the proliferation of hypoxia colon cancer cells, colon cancer cells were divided into normoxia group, hypoxia control group and PLD2 activity inhibit group(50 nM and 100 nM). The activity of PLD2 and the proliferation of all cells of four groups were detected by PLD activity assay and MTT assay, respectively. As a result, PLD2 activity was significantly decreased by VU0364739 compare to hypoxia control group (Fig. 2a, $P < 0.05$). As expected, inhibit the activity of PLD2 also significantly reduce the proliferation of colon cancer cells compare to hypoxia control group (Fig. 2b, $P < 0.05$), it present concentration dependent manner. These results suggested PLD2 promotes the proliferation of colon cancer cells under hypoxic conditions.

PhospholipaseD2 Promotes the Proliferation of Colon Cancer Cell through Up-Regulates the Expression of Cyclin D1 by Activating NF-κBp65 Signaling Pathway under Hypoxic Conditions

High expression of NF-κBp65 is associated with cell proliferation and poor clinical outcome in colon cancer [14]. Furthermore, activated NF- κBp65 expression may play anticancer role in human cancers [15]. PLD has been reported that regulates the proliferation of cells through regulates the expression of Cyclin D1 [16]. Therefore, we detected the expression level of p-p65/ NF-KBp65 and Cyclin D1 in cells of every group. Our results showed that hypoxia induced the p-p65/NF-KBp65 protein and NF-KBp65 mRNA expression level increased compare with normoxia group, as well as the protein and mRNA expression level of Cyclin D1 were increased, they all have significantly difference between hypoxia group and normoxia group ($P<0.05$). However, PLD2 down-regulation or PLD2 activity inhibition significantly reduced the proteins expression levels of p-P65/NF-κBp65 and Cyclin D1 (Figs. 3a and 4a) in colon cancer cells, as well as the mRNA level of NF-κBp65 and Cyclin D1 decreased (Figs. 3b and 4b). Those results suggesting that PLD2 promote the proliferation of colon cancer cell may partly through up-regulate the expression of Cyclin D1 by activating NF-κBp65 signaling pathway.

In order to further confirm our results, the inhibitor of NF-κBp65 BAY-117082 was used. Colon cancer cells were divided into hypoxia control group and NF-κBp65 inhibit group. We found that BAY-117082 could decreased the protein expression level of p-P65/NF-κBp65 and Cyclin D1 in hypoxia colon cancer cells (Fig. 5a, b), as well as the mRNA expression level of Cyclin D1 decreased in those cells (Fig. 5c), while the rate of proliferation decreased (Fig. 5d). These results indicated that high expression of PLD2 induced by hypoxia promotes the proliferation may part due to up-regulates the expression of CyclinD1by NF-κBp65 activation.

Down-Regulation of PLD2 Significantly Reduces Tumor Volume and the Expression of NF- κ Bp65 and Cyclin D1 In Vivo

In vivo growth study was performed by SW620 and SW480 cells. We set up xenograft tumor model according to the method introduce in methods and materials. The growth of the xenograft tumor was studied by tumor growth curve. Expression of P65/NF- κ Bp65 and Cyclin D1 in xenograft tumor was detected by western blot analysis. From the graph of tumor growth curve, we found that there was statistical difference in tumor growth between PLD2 interference group and control group after 18 days (Fig. 6a,

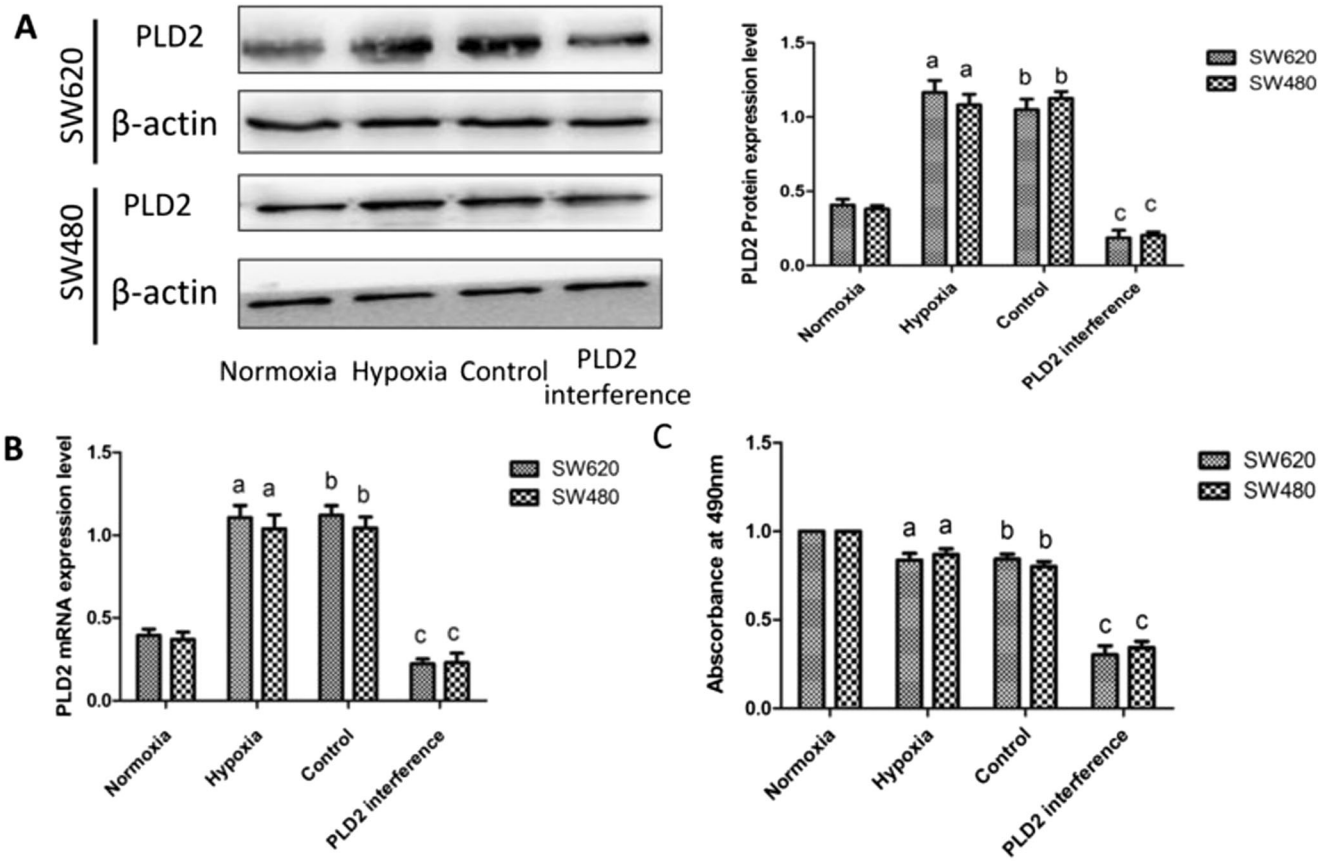

Fig. 1 Decreased the expression level of PLD2 leaded to the proliferation of colon cancer cells reduced under hypoxic conditions. **a** and **b** Hypoxia induce the protein and mRNA expression level of PLD2 increased, while ad-PLD2-siRNA significantly reduced the expression level of PLD2 compare with hypoxia control group; **c** Hypoxia induced the proliferation of colon cancer cells decreased, while decreased the expression of PLD2 leaded to the proliferation of colon cancer cells further decreased. (a $p < 0.05$vs Normoxia group; b p>0.05vs hypoxia group; c $P < 0.05$vs hypoxia control group)

$p < 0.01$). Moreover, it's interesting that the growth of xenograft tumor had no difference between blank control group and normal control group at the same stage ($P > 0.05$). As shown in Fig. 6b, the volume of xenograft tumor in PLD2 interference group is significant smaller than control group ($p < 0.01$), but there is no difference between blank control group and normal control group ($p > 0.05$). From western blot analysis (Fig. 6c), we found that the expression level of P65/NF-KBp65 and Cyclin D1 in PLD2 interference group was there was significant lower than control groups ($p < 0.01$), but there is no difference between blank control group and normal control group ($p > 0.05$). Those results further confirmed that PLD2 promotes the proliferation of hypoxia colon cancer cells may through up-regulates the expression of Cyclin D1 by activating NF-κBp65 signaling pathway.

Discussion

In this study, we found that hypoxia induce the proliferation of colon cells reduced. However, decreased the expression of PLD2 or inhibition of the activity of PLD2 both lead to the proliferation colon cancer cells further reduced under hypoxic conditions in vivo or in vitro. An accumulation of evidence confirms PLD2 as a regulator of cell fate including cancer cells [17]. Zhao Yutong et al. found that PLD2 is overexpressed to a great extent in human kideny cancer tissue suggesting that PLD2 has an effect on proliferation and may be related to cancer development [18]. Those results suggest that PLD2 may promote the proliferation of colon cancer cells under hypoxic conditions.

To reveal the molecular mechanism underlying PLD2 regulates colon cancer proliferation, we further detected signaling molecules involved in cell growth and survival in colon cancer cells after PLD2 gene interference or PLD2 activity inhibited. Dong Woo Kang et al. reported that positive feedback regulation between PLD and Wnt signaling promotes the growth of colorectal cancer cell [19]. Previous studies show that PLD2 mediated the signaling from the members of growth factors and regulate receptor-induce cell survival [20]. In gliobalstoma cells, PLD2 mediate surviving signaling through regulating of AKT pathway [21]. Furthermore, one of the targets of PLD signaling is mammalian target of rapamycin (mTOR), which is a critical

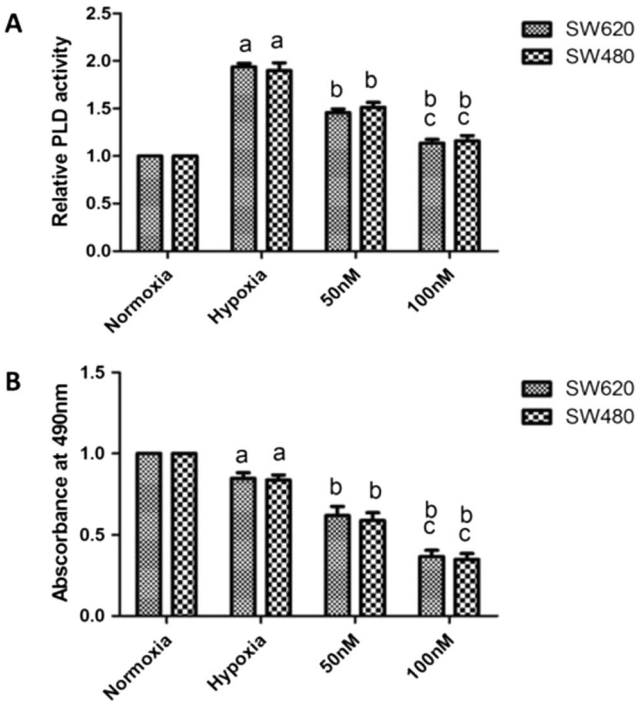

Fig. 2 Inhibition the activity of PLD2 lead to the proliferation of colon cancer cells reduced under hypoxic conditions. **a** Hypoxia induced the activity of PLD2 elevated, while VU0364739 lead to the activity of PLD2 induced by hypoxia decreased. **b** Hypoxia induced the proliferation of colon cancer cells decreased, while inhibition the activity of PLD2 lead to the proliferation of hypoxia colon cancer cells decreased. (a $p < 0.05$vs Normoxia group; b $p>0.05$vs hypoxia group; c $P < 0.05$vs hypoxia control group)

regulator of cell cycle progression and cell growth [22]. Which suggest that PLD2 may influence the growth of cancer through many signaling pathway. NF-κBp65 has been well proved to be associated with cell proliferation. NF-κBp65 high expression is associated with cell proliferation and poor clinical outcome in colon cancer [22]. Furthermore, inactive NF-κBp65 expression may play anticancer role in human cancers [23]. PLD2 catalytic hydrolysis phosphatidylcholine to PA and chonline, which can take part in the signal transduction in many cancer cells. PA also may trigger downstream signaling cascades including NF-kB activation and then regulate transcription of a number of target related to tumorigenesis and cell survival [23]. Mi Hee Park et al. also reported over-expression of PLD enhances MMP-2 expression via NF-kB signaling pathway [24]. So we inferred that PLD2 may mediates the growth of colon cancer cell through activating NF-kB signaling pathway. In our study, we observed that interference PLD2 gene expression or inhibition of the activity of PLD2 decreased the protein expression level of p-p65/ NF-KBp65 in colon cancer cells under hypoxic conditions. Those results indicated that PLD2 promotes the proliferation of colon cancer cells under hypoxic conditions may through activating NF-KBp65 signaling pathway, and then up-regulates the expression of Cyclin D1. To further confirm this result, we inhibition of NF-KBp65 pathway by BAY117082 and the proliferation of colon cancer cells under hypoxic conditions was examined by MTT assay. The results showed that, inhibition of expression level of p-p65/ NF-KBp65 lead to the proliferation of those cells reduced, as well as the expression level of Cyclin D1decreased. The result suggests that PLD2 may promote the proliferation of colon cancer cells may through up-regulates the expression of Cyclin D1 via activating NF-κBp65 signaling pathway. However, activation PI3K/AKT [25] and PKC/PKA [24] signaling pathway have been suggested to be involved in PLD2 activate NF-κBp65 and then take part in the course of kidney cancer and gliobalstoma carcinogenesis. Whether PLD2 promote the growth of colon cancer cells through those signaling pathways then activate NF-κBp65 pathway needed to be further studied.

In conclusion, these results suggest that the function of PLD2 may be multiple, multifarious and multifaceted, and may influence tumorigenesis through multiple ways. Apart from others signaling pathway that promote colon cancer growth, we show that PLD2 promotes the growth of colon cancer cell may activating NF-κBp65 signaling pathways here. Therefore, inhibition of PLD2 may have advantages over the present signaling pathway inhibitors in PLD2-overexpressed colon cancer patients.

Materials and methods

Cell Culture and Reagents

Human colon cancer cell lines (SW480,SW620) were purchased from the Shanghai Cell Bank at the Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in RPMI 1640 medium (Gibco, GrandIsland, NY) supplemented with 10% fetal bovine serum (Hyclone, Shanghai, China) and 1% penicillin/streptomycin (Beyotime, Jiangsu, China) at 37 °C in a humidified incubator containing 5% CO_2. Hypoxia culture performed in a three-chamber air incubator with the flushed with a gas mixture of 5%CO_2–94%N_2 at 37 °C,the final O2 press value of the medium was continuous measured at a range of 0.5–1%.

Primary antibodies of PLD2, Cyclin D1,NF- κ Bp65 and p-P65 were purchased from Abcam Biotechnology. The inhibitor of NF- κ Bp65 (BAY117082) and PLD2 activity inhibitor (VU0364739) were purchased from sigma and Santa Cruz Biotechnology, respectively.

Transfection of Colon Cancer Cells with Interference RNA

Human-specific PLD2 interference RNA (siRNA) was designed, constructed and purified by Hanheng Biotech

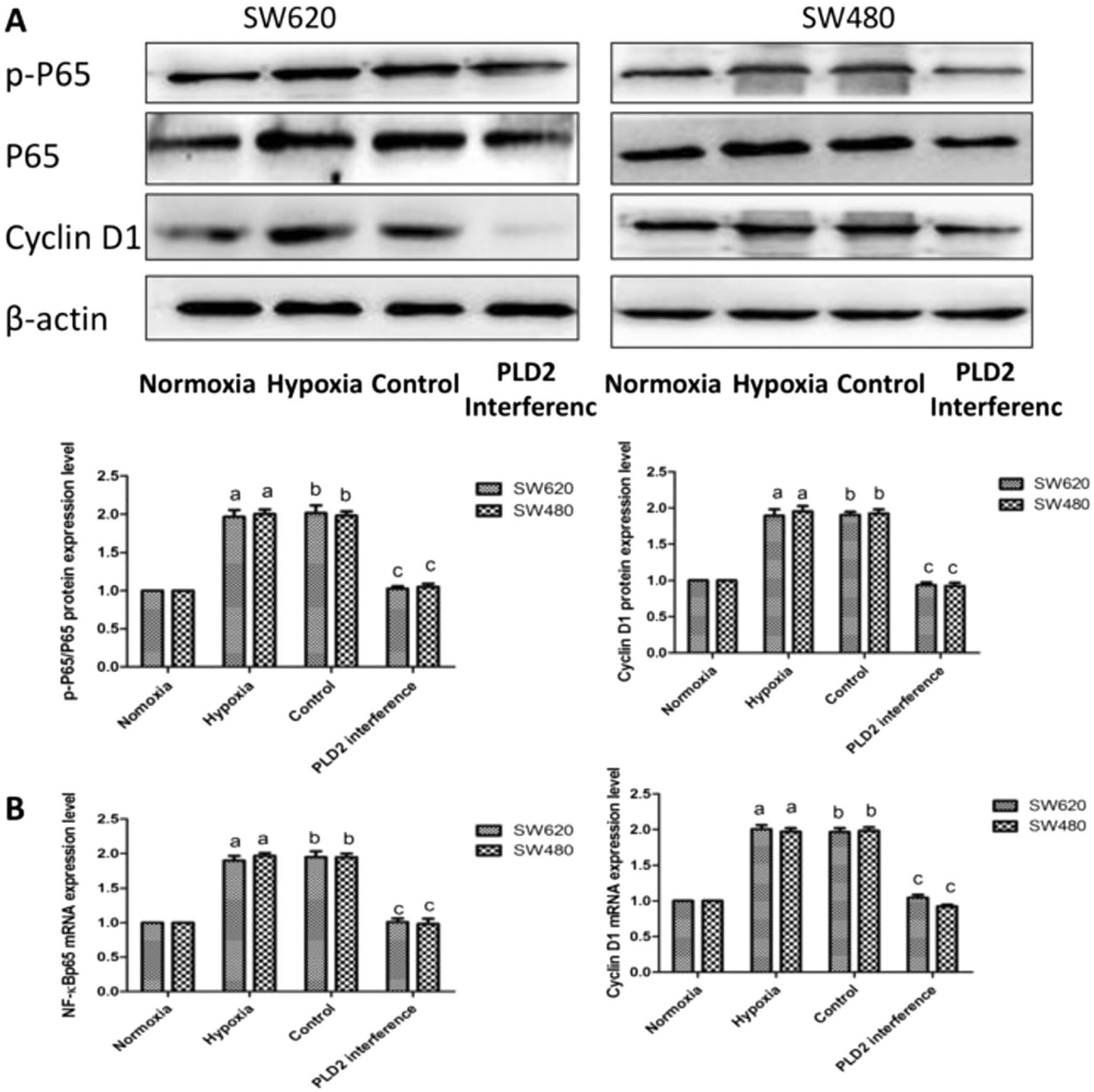

Fig. 3 Decreased the expression level of PLD2 leaded to the expression of p-P65/NF- κ Bp65 and Cyclin D1 in colon cancer cells reduced under hypoxic conditions. **a** Protein expression level of p-P65/NF- κ Bp65 and Cyclin D1 increased compare to normoxia group, while decreased the expression level of PLD2 lead to the protein expression level of p-P65/NF- κ Bp65 and Cyclin D1 reduced compare with hypoxia control group. **b** The mRNA expression level of NF- κ Bp65 and Cyclin D1 increased compare to normoxia group, while decreased the expression level of PLD2 lead to the mRNA expression level of NF- κ Bp65 and Cyclin D1 reduced compare with hypoxia control group. (a $p < 0.05$ vs Normoxia group; b $p > 0.05$vs hypoxia group; c $P < 0.05$ vs hypoxia control group)

Co(Shanghai, China). PLD2 siRNA composed of sense 5'-ACAUAAAGGUGAUGCGUCA -3′. After determining the best effect of interference and the best transfer multiplicity of infection (MOI) through fluorescence camera, RT-PCR and Western-blot, SW480 and SW620 cells were seeded in six-well plates at a concentration of 0.5×10^5 per well(20–30% confluence) on the day before ad-PLD2- siRNA transfection.

Next day, the ad-PLD2-siRNA was transfect into cells (interference group) at a MOI of 50 (SW620) or 60 (SW480). Simultaneously, the blank vector of adenovirus was transfected into cells as negative control for control the impact of adenovirus vector. After incubation for 4 h, medium was replaced with fresh RI-1640. Transduction effects were observed with a fluorescence

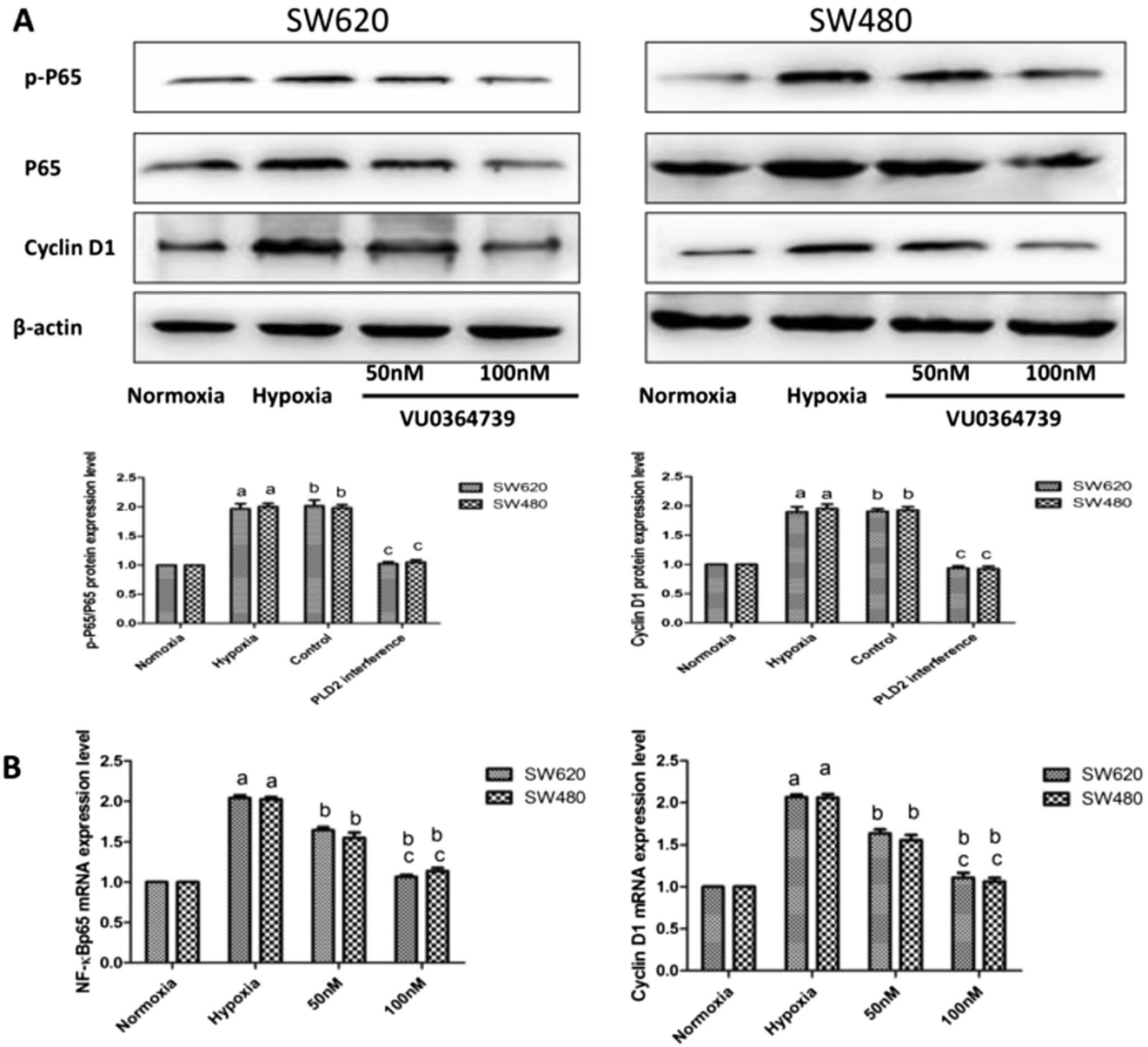

Fig. 4 Inhibition the activity of PLD2 lead to the expression of p-P65/NF-κBp65 and Cyclin D1 of colon cancer cells reduced under hypoxic conditions. **a** High protein expression of p-P65/NF- κ Bp65 and Cyclin D1 were decreased when the activity of PLD2 was inhibited. **b** High mRNA expression of NF- κ Bp65 and Cyclin D1 were decreased when the activity of PLD2 was inhibited. (a $p < 0.05$vs Normoxia group; b $p > 0.05$vs hypoxia group; c $P < 0.05$vs hypoxia control group)

microscopy camera 24 h after transduction. All the cells were cultured under hypoxic conditions for 24 h and harvested for subsequent studies for mRNA and protein as well as other assays.

PLD Activity Assay

PLD activity was determined as previously describe, by measuring [^{3}H] phosphatidyl ethanol (PEt) produced via PLD-catalyzed transphosphatidylation in [^{3}H] palmitic acid-labeled cells [26].

Quantitative Transcription-Ploymerase Chain Reaction(qRT-PCR) Analysis

Total RNA of the cell lines was extracted using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. After evaluating the concentration and purity of total RNA by using an UV spectrophotometer (Utrospec2100 pro, Amersham, USA), the total RNA was reverse-transcribed. Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II (TakaRa, Dalian, China) with CFX96™ Real-Time System (BIO-RAD, USA), with each sample analyzed

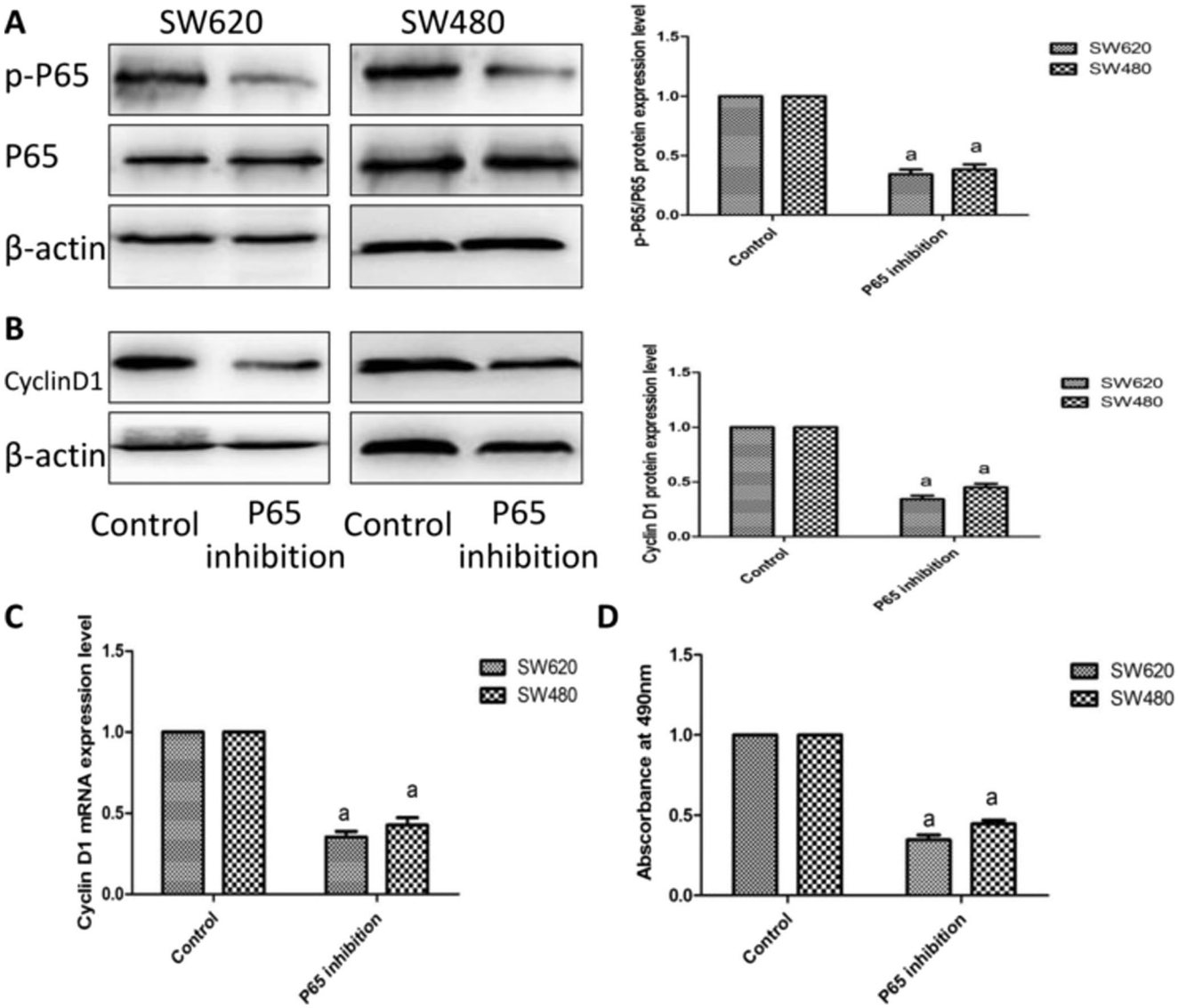

Fig. 5 Inhibition of NF- κ Bp65 signaling pathway lead to the proliferation and expression level of Cyclin D1in hypoxia colon cancer cells decreased. **a** Protein expression level of p-P65/NF-κBp65 was significant reduced compare with control group; **b** and **c** Inhibition the expression level of p-P65/NF-κBp65 leads to the protein and mRNA expression level of Cyclin D1 reduced significantly compare with control group. **d** Inhibition the expression level of p-P65/NF- κ Bp65 leads to the proliferation of colon cancer cells reduced compared with control group. (a $p < 0.05$vs Control group)

in triplicate. Relative levels of mRNA expression were normalized for β-actin mRNA expression (as internal control) and calculated according to the formula $2^{-(Ct\ sample-Ct\ control)}$ as previous described [27]. Primer sequence for the genes analyzed as follows:

PLD2:forward5'-GTGGGCGATGAGATTGTGGAC-3′, reverse5'-CAGGATTGAATACCCCCACGA-3'; NF-κBp65:forward5'-GGGAATGGTGAGGTCACTCTAA–3′, reverse5'-AATGAAGGTGGATGATTGCTAAG-3'; CyclinD1:forward5'-CTGGCCATGAACTACCTGGA-3′, reverse5'-GTCACACTTGATCACTCTGG-3′. β-actin:forward5'-CCACGAAACTACCTTCAACTCC-3′, reverse5'-GTGATCTCCTTCTGCATCCTGT-3.

Western Blot Analysis

Total proteins were extracted from harvested cells by protein extract kit (Beyotime, Jiangsu, China). All the proteins were degenerated by boiling, separated by SDS-PAGE gel and transferred onto PVDF membranes. Then, membranes were blocked by 5% milk. Next, the membranes were incubated by first antibodies overnight at

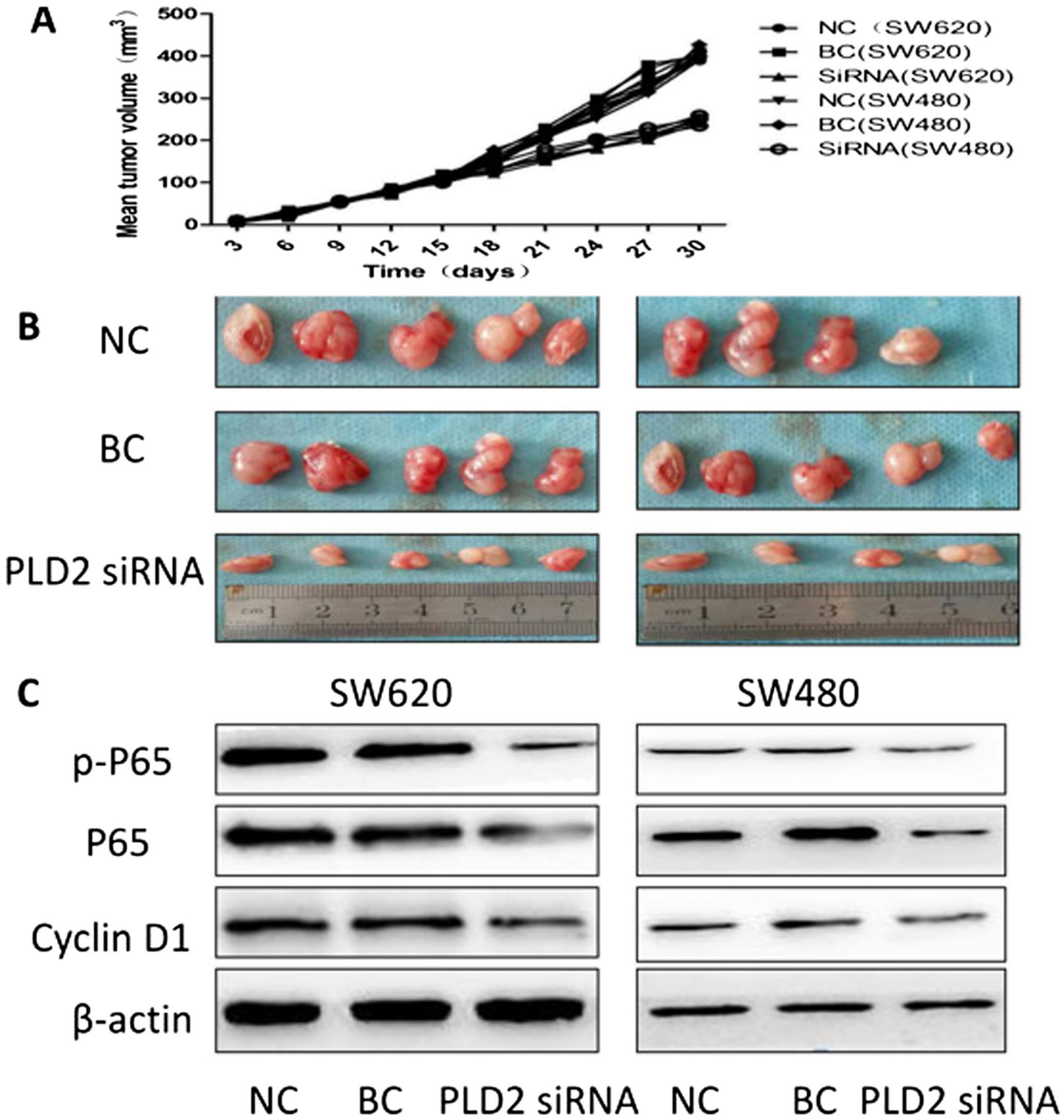

Fig. 6 Down regulation of PLD2 significantly reduces tumor volume and the expression level of p-P65/NF-κBp65 and Cyclin D1 in vivo. **a** Tumor volume was monitored over time. **b** Representative xenograft tumors are shown. **c** Western blot analysis the expression of p-P65/NF-κBp65 and Cyclin D1 in xenograft tumor

4 °C. The next day, after washed by TBS contain 0.05% Tween-20, the membranes were incubated by second antibody (goat anti-rabbit) for 2 h under 37 °C incubator. Finally, all the membranes were visualized by a chemiluminescence kit (Beyontime, Jiangsu, China) on a Bio-Rad imaging system. The band intensities were quantified using Image J software (Vilber FUSION FXS Spectra, France). Primary antibodies used in this study are as follows:PLD2(1:1000, Abcam Biotechnology, Cambridge, UK); Cyclin D1(1:1000, Abcam Biotechnology, Cambridge, UK); NF-KBp65(1:1000, AbcamBiotechnology, Cambridge, UK); p-p65(1:750, Abcam Biotechnology, Cambridge, UK) and β-actin (1:500, Boster, biotechnology, Wuhan, China).

Cell Proliferation Assay

Cell viability which is an index of cell proliferation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT)(sigma, St, Louis,USA),according to the manufacturer's instructions. Briefly, SW620 and SW480 cells were trypsinized and seeded in 96-well plates at 5×10^4 cells per well. All cells of every group were plated with five replicates for each group. After transfecting ad-PLD2-siRNA or blank adenovirus, cell viability was detected using an MTT assay as follows: 20 μl of MTT solution(5 μg/μl) was added to each well and incubated for 4 h, the cultural media was removed and replaced with 150 μl of DMSO, and vibrated for 10 min on the shaking table. After mixing, the absorbance (OD) of the cells was measured at 490 nm by using an enzyme standard instrument. Growth curves were evaluated with time points as the abscissa and OD as the vertical coordinate.

Animal Study

Five-week old female BALB/C nude mice were purchased from the Laboratory Animal Center of Shanxi Medical University and were maintained in specific pathogen-free units under isothermal conditions. All experimental procedures were carried out in accordance with the national institute of Health Guide for the care and use of laboratory animals. SW620 and SW480 cells (5×10^6) were subcutaneous injected. Measured the length(L) and width(S) of the tumor every three day. After the volume of tumor was get to 90–120 mm^3, which calculated according the formula $V = 0.5 \times L \times S^2$, the animals were divided into three groups including interference group, blank control group and normal control group. After first time injected, one times every two days, eight times altogether. Then animals were sacrificed and the tumor was dissected for further study.

Statistical Analysis

All the experiments were repeated in triplicate. Analysis was carried out using SPSS17.0 statistical software (SPSS, Inc., Chicago, IL, USA) and data are displayed as the mean ± standard deviation (SD). Student's t test or one-way ANOVA was used to analysis quantitative data. x^2-test was used to analysis count data. Differences were considered significant at $p < 0.05$.

Conclusions

High expression of PhospholipaseD2 induced by hypoxia promotes proliferation of colon cancer cells through activating NF- κ Bp65 signaling pathway.

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Author Contributions Bo Jiang and Xingye Wu conceived and designed the experiments; Maoxi Liu and Kunli Du performed the experiments; Maoxi Liu analyzed the data and wrote the paper.

Compliance with ethical standards

Conflicts of Interest The authors declare no conflict of interest.

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