CBX3 promotes ovarian cancer progression by regulating p53/p21-mediated glucose metabolism via inhibiting NCOR2

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Abstract

Introduction: Chromobox protein homolog 3 (CBX3) has been reported to play an oncogenic role in various tumors. Nevertheless, the role of CBX3 in ovarian cancer remains vague.
Material and methods: Chromobox protein homolog 3 expression was determined by qRT-PCR and western blotting in ovarian cancer tissues and cell lines. Cell proliferation, cycle and apoptosis were detected using CCK-8 assay and flow cytometry. Transwell and wound healing assays were used to determine cell invasion and migration. Furthermore, the modulatory effects of CBX3 on NCOR2 expression and p53/p21-mediated glycolysis were confirmed.
Results: The expression of CBX3 was significant elevated in ovarian cancer tissues and cell lines. CBX3 knockdown inhibited cell proliferation, invasion and migration, while it promoted G1/S phase blockade and cell apoptosis. Mechanism analysis verified that CBX3 downregulation increased NCOR2 expression and blocked subsequent p53/p21-mediated glycolysis. NCOR2 silencing and p53/p21 inhibitor treatment reversed the inhibitory effects of CBX3 knockdown on ovarian cancer cellular function.
Conclusions: CBX3 promoted ovarian cancer progression by promoting p53/p21-mediated glycolysis via inhibiting NCOR2.

Key words: CBX3, NCOR2, p53/p21, glycolysis, ovarian cancer.

Introduction

Ovarian cancer (OC) is a common gynecological malignancy worldwide, and its mortality rate ranks forefront among gynecological tumors [1]. Emerging evidence has proved that molecular targeted therapy may be an effective treatment method. However, the molecular mechanism of OC still remains unclear, which limits the progress of clinical treatment. Therefore, identification of novel biomarkers and elucidation of their mechanisms are critical for the diagnosis and therapy of patients with ovarian cancer.

Chromobox protein homolog 3 (CBX3, also called HP1γ), a member of the heterochromatin protein 1 family, is involved in regulation of a va-

riety of cellular biological functions [2]. Multiple previous studies have reported that CBX3 was abnormally expressed and regulated cellular functions in a variety of cancer diseases [3–5]. In pancreatic cancer, CBX3 also has been reported to be associated with glycolysis [6]. A recent study suggested that abnormally high expression of CBX3 was found in ovarian cancer tissues [7]. However, the precise role and regulatory mechanism of CBX3 in ovarian cancer remain unclear.

Alteration of glucose metabolism is a faster but less efficient process than mitochondrial oxidative phosphorylation in ATP production and is considered as a hallmark of cancer [8, 9]. In the tumor microenvironment, most cancer cells showed increase of glucose consumption and lactate secretion, which promotes cancer environmental adaptability and cell survival [10]. Subsequently, the decreased potential of hydrogen (PH) caused by elevated glucose uptake and lactate production promotes epithelial-to-mesenchymal transition (EMT), ultimately facilitating cancer cell migration and invasion [11]. Alteration of glucose metabolism in cancer cells is driven by inactivation of tumor suppressors, including p53 [12].

In the present study, the role of CBX3 on cell growth and glycolysis was investigated, and the putative regulatory mechanism of CBX3 in ovarian cancer was further explored. The results provide a novel biomarker for the diagnosis and therapy of patients with ovarian cancer.

Material and methods

Ovarian cancer samples

The 30 pairs of epithelial OC tumor tissue and corresponding adjacent non-tumor tissue (about 3 cm away from the tumor tissues) samples were obtained from OC patients undergoing surgery at the General Hospital of Ningxia Medical University. All samples were histopathologically diagnosed and confirmed by a pathological evaluation. Informed consent was obtained from all patients. Ethics approval was given by the General Hospital of Ningxia Medical University (KYLL-2022-0271). All procedures conformed to the Declaration of Helsinki.

Cell culture and transfection

Human normal ovarian epithelial cell line IOSE80 and OC cell lines (ES2, SKOV3, OVCAR3, A2780, HEY) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China) and kept in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., MA, USA), penicillin (100 µg/ml) and streptomycin (100 µg/ml) (Thermo Fisher Scientific, Inc., MA, USA) with 5% CO_2 at 37°C.

All indicated siRNA (siCBX3, siNCOR2), pcDNA3.1 (pcNCOR2) and the respective negative control (siCtrl, pcCtrl) were synthesized and constructed by Shanghai GenePharma Company (China). Transfection into SKOV3 cells was performed using Lipofectamine 2000 (Invitrogen, Waltham, USA). All the transfected cells were collected at 48 h after transfection for the following experiments.

qRT-PCR

RNA was extracted from tissue samples and cultured cells using Trizol reagent (TaKaRa, Shiga, Japan). The reverse transcription of extracted RNA was synthesized using a Primer Script RT Reagent Kit (TaKaRa, Shiga, Japan). PCR amplification was performed using SYBR Premix Ex Taq (TaKaRa, Shiga, Japan). The total volume of the reaction mixture was 20 µl, which contained 1 µl of each primer, 2 µl of cDNA and 10 µl of SYBR Premix Ex Taq. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an internal standard. Relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Protein was extracted and quantified using a BCA kit (Pierce, Shanghai, China). Then, protein samples were separated by SDS-PAGE electrophoresis and transferred onto PVDF (Millipore, Billerica, MA, USA) membranes. After being blocking with 5% nonfat milk, the membranes were incubated with primary antibodies CBX3 (1:1000), cyclin A (1:1000), cyclin B1 (1:2000), cyclin D1 (1:1000), cyclin E (1:1000), Bcl-2 (1:1000), BAX (1:1000), caspase-3 (1:500), E-cadherin (1:10000), N-cadherin (1:100), vimentin (1:1000), NCOR2 (1:1000), p53 (1:1000), p21 (1:1000), and p-p53 (1:1000) at 4°C overnight separately. Rabbit anti-human GADPH (1:2500) was used as an internal control. Subsequently, the membranes were washed three times and incubated with horseradish peroxidase-labeled secondary antibody (Sigma, St. Louis, MO, USA) for 1 h. Proteins were visualized by an enhanced chemiluminescence detection system (ECL, Thermo Fisher Scientific, USA). The blots were quantitatively analyzed using Image Lab 3.0 (Bio-Rad, USA).

CCK-8 assay

CCK-8 reagents (Dojindo, Japan) were used to detect cell proliferation according to the manufacturer's protocol. Cells (5×10^3 cells/well) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum for 24 h. Then, cells were washed and starved for 24 h. After 48 h incubation, 10 µl of CCK-8 was added to each

well to culture for 2 h. Finally, the plates were placed in a microplate reader (Thermo Fisher Scientific, USA) for absorbance determination for 450 nm.

Flow cytometry analysis

The cell cycle and cell apoptosis were detected using a cell cycle kit (US Everbright Inc) and an Annexin V fluorescein isothiocyanate kit (FITC; BD, USA). An FC-500 flow cytometer (Beckman Coulter, Brea, USA) was used to evaluate the cell cycle distribution and percentage of apoptotic cells.

Wound healing assay

Cells were incubated at 37°C with 5% CO_2 until they reached 90% confluence. After starvation, cells (1×10^6 cells/well) were wounded with sterile 200 µl pipette tips, and the injury line were marked. Then, the disconnected cells were washed with PBS, while the remaining cells were incubated with RPMI-1640 medium supplemented with 10% FBS. Micrographs were acquired at 0 and 24 h.

Invasion assay

Cell invasion assays were performed using Transwell chambers (8 µm pore size) (BD, USA). Cells (1×10^5 cells/well) in serum-free medium were seeded in the upper chamber coated with Matrigel (1:6; BD, USA) before. Medium supplemented with 20% FBS was added to the lower chambers, and cells were cultured in a thermostatic incubator with 5% CO_2 at 37°C for 24 h. Finally, invaded cells were stained with 0.5% crystal violet and counted under a microscope (Olympus Crop., Tokyo, Japan).

Detection of ATP level and oxygen consumption

An ATP Determination Kit (Thermo Fisher Scientific) was used to assess the ATP production. Briefly, ovarian cancer cells were lysed, centrifuged and filtered. Then, treated samples were mixed with ATP reaction mix. A Glomax 20/20 (Promega, USA) luminometer was used to determine the ATP production. Extracellular Oxygen Consumption Assay (Abcam) was introduced to determine oxygen consumption.

Measurement of glucose consumption and lactate production

After transection, ovarian cancer cells were harvested and centrifuged to collect the supernatant of cells. A Glucose and Lactate assay kit (BioVision, Milpitas, CA, USA) was used to detect the amount of glucose and lactate.

Measurement of glucose-6-phosohate (G6P) levels

A G6P assay kit with WST-8 (Beyotime, Shanghai, China) was used to evaluate the level of G6P in cells according to the manufacturer's instructions.

Statistical analysis

The results are presented as mean ± SEM of at least three independent experiments. Statistical analyses were performed using GraphPad Prism software version 8.2 (La Jolla, CA, USA). The results were consistent with the normal distribution. The pairwise comparisons were performed using Student's t-test and the comparisons among multiple groups were performed by the one-way ANOVA analysis test. $P < 0.05$ (*) indicates statistically significant differences.

Results

CBX3 expression was increased in OC tissues and cell lines

The results showed significant up-regulation of CBX3 in OC tissues compared with the adjacent non-tumor tissues (Figure 1 A). Consistently, increased expression of CBX3 mRNA and protein was also detected in OC cell lines compared with IOSE80 cell lines (Figures 1 B, C). In addition, CBX3 presented the highest expression level in SKOV3 cell lines, so the SKOV3 cell line was used in subsequent experiments.

Knockdown of CBX3 suppressed OC cell proliferation while promoting G1/S phase blockade and cell apoptosis

As expected, the mRNA and protein expression levels of CBX3 were reduced in si-CBX3s transfected cells (Figure 2 A, B). Knockdown of CBX3 conspicuously suppressed cell proliferation in SKOV3 cells (Figure 2 C). Also, inhibition of CBX3 led to cell arrest at G1/S phase, resulting in decreased cell numbers at S phase, accompanied with lower expression of cyclin A, cyclin B1, cyclin D1 and cyclin E (Figures 2 D, E). Furthermore, CBX3 silencing promoted cell apoptosis as evidenced by elevated Bax and cleaved caspase 3 expression and reduced Bcl-2 expression (Figures 2 F, G).

Knockdown of CBX3 suppressed OC cell invasion and migration

Down-regulation of CBX3 significantly suppressed cell invasion of SKOV3 cells (Figure 3 A). Moreover, wound healing assay showed that knockdown of CBX3 also dramatically inhibited cell migration (Figure 3 B). In addition, epitheli-

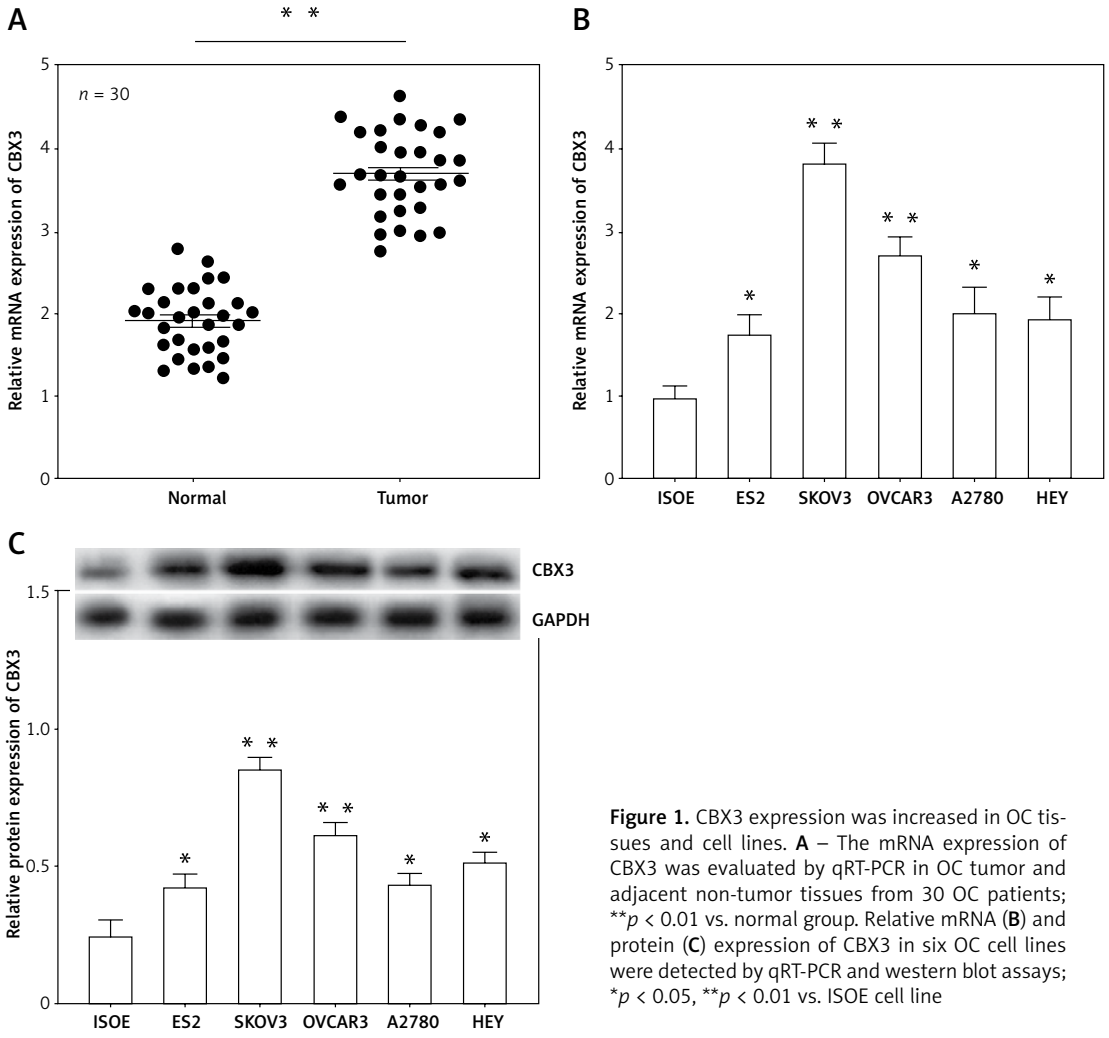

Figure 1. CBX3 expression was increased in OC tissues and cell lines. **A** – The mRNA expression of CBX3 was evaluated by qRT-PCR in OC tumor and adjacent non-tumor tissues from 30 OC patients; $^{**}p < 0.01$ vs. normal group. Relative mRNA (**B**) and protein (**C**) expression of CBX3 in six OC cell lines were detected by qRT-PCR and western blot assays; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. ISOE cell line

al-mesenchymal transition (EMT) is widely recognized as a marker of tumor cell metastasis. CBX3 silencing markedly increased the expression of the epithelial regulator E-cadherin, and decreased the expression of the mesenchymal regulators N-cadherin and vimentin (Figures 3 C, D).

Knockdown of CBX3 suppressed glycolysis of OC cells

The results showed that CBX3 knockdown markedly suppressed glucose upstate, lactate production and G6P level in SKOV3 cells (Figures 4 A–C). Also, the results showed that downregulation of CBX3 significantly inhibited ATP production and O_2 consumption (Figure 4 D, E).

CBX3 blocked activation of the p53/p21 pathway by inhibiting NCOR2

Notably, we found that NCOR2 was downregulated in OC tissues and cell lines (Figures 5 A–C). Meanwhile, CBX3 knockdown promoted the expression of NCOR2 (Figure 5 B, C). Correlation analysis showed that CBX3 negatively correlated with NCOR2 expression in OC tissues (Figure 5 D). Subsequently, we transfected siNCOR2 and pcNCOR2 to reduce and boost the expression of NCOR2 in OC cells (Figures 5 E, F). The protein expression levels of p53, p21 and p-p53 were increased after transfecting siCBX3-1 or pcNCOR2. These results were rescued by co-transfecting with siCBX3-1 and siNCOR2 in SKOV3 cells (Figure 5 G).

CBX3 mediated glycolysis by blocking p53/p21 via inhibiting NCOR2

The results showed that CBX3 knockdown facilitated ATP production and hindered glucose uptake, lactate production and G6P level; these effects were abolished by NCOR2 knockdown and pifithrin-α (PFT-α; p53/p21 inhibitor) treatment (Figures 6 A–D). Moreover, the up-regulation of NCOR2 also promoted ATP production and reduced glucose uptake, lactate production and G6P level, which were restored by inactivating the p53/p21 pathway (Figures 6 A–D).

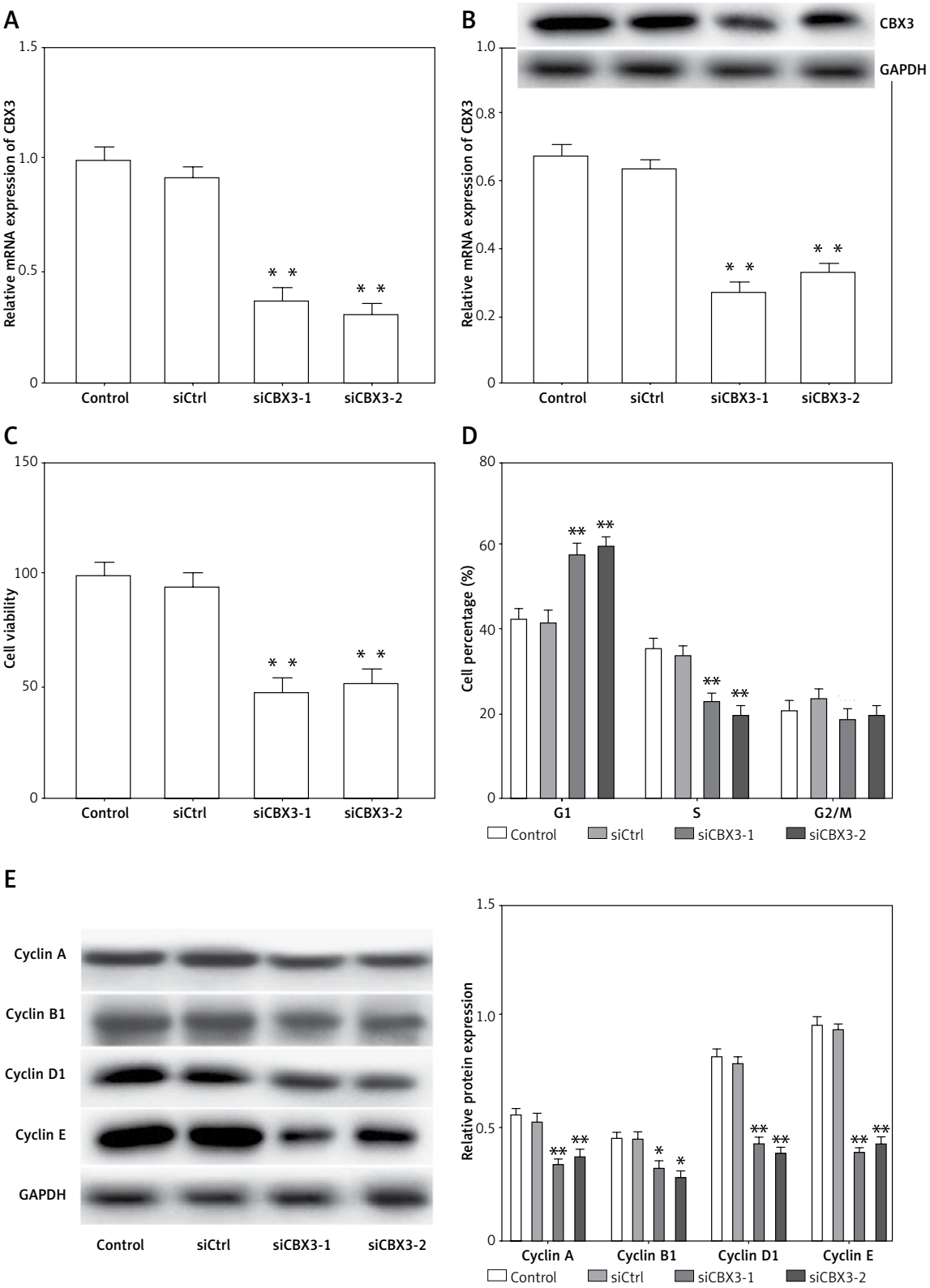

Figure 2. Knockdown of CBX3 suppressed OC cell proliferation while promoted G1/S phase blockade and cell apoptosis. Relative mRNA (**A**) and protein (**B**) expression of CBX3 were confirmed by qRT-PCR and western blot assays. **C** – Cell viability was detected by CCK-8 assay in SKOV3 cells after transfection with siCBX3s. **D** – Flow cytometry analysis for cell cycle in SKOV3 cells after transfection with siCBX3s. **E** – Relative cell cycle related protein expression were examined by western blot assay

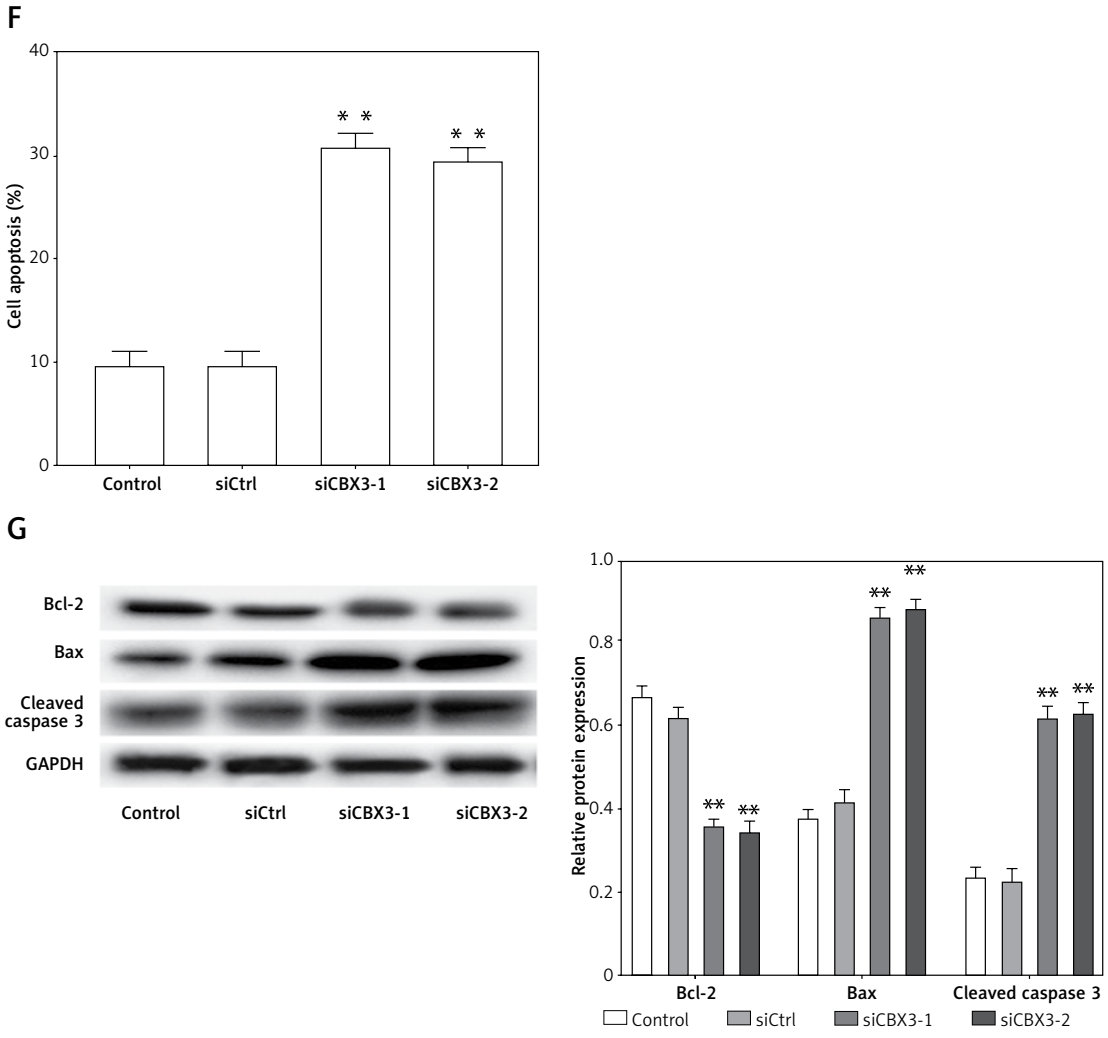

Figure 2. Cont. **F** – Flow cytometry analysis for cell apoptosis in SKOV3 cells after transfection with siCBX3s. **G** – Relative cell apoptosis related protein expression were detected by western blot assay; $^{**}p < 0.01$ vs. control group

CBX3 regulated OC progression by blocking the p53/p21 pathway via inhibiting NCOR2

The results showed that knockdown of CBX3 markedly inhibited cell proliferation and the cell cycle, and promoted cell apoptosis, which were reversed by siNCOR2 and PFT-α (Figures 7 A–E). Furthermore, knockdown of NCOR2 and treatment with PFT-α significantly rescued the effects on cell invasion and migration concomitant with the increased E-cadherin expression and decreased N-cadherin and vimentin expression in CBX3-downregulated OC cells (Figures 7 F–H). Similarly, PFT-α also alleviated the impacts of NCOR2 overexpression on OC cell proliferation, the cell cycle, apoptosis, invasion and migration (Figures 7 A–H).

Discussion

Ovarian cancer is considered as the most lethal gynecological malignancy owing to the lack of early diagnosis and effective treatment [13]. Therefore, understanding the molecular mechanism of ovarian cancer initiation and development is vital. Previous studies have reported that elevated expression of CBX3 was closely related to the progression of various cancers. CBX3 was reported to promote colorectal cancer proliferation via targeting p21 [3]. In gastric cancer, knocking down CBX3 resulted in inhibition of proliferation, invasion and cell cycle arresting at G1 phase [14]. Moreover, CBX3 was considered as a novel therapeutic strategy in pancreatic adenocarcinoma [4]. In this study, the results showed that CBX3 was upregulated in OC tissues and cell lines. Xu and colleagues demonstrated forced expression of CBX3 protein by detecting with immunohistochemistry in ovarian carcinoma tissues, which is consistent with these findings [7]. Importantly, we found that knockdown of CBX3 suppressed cell proliferation, invasion and migration, and also led to cell cycle arrest at G1/S phase and promoted cell apoptosis.

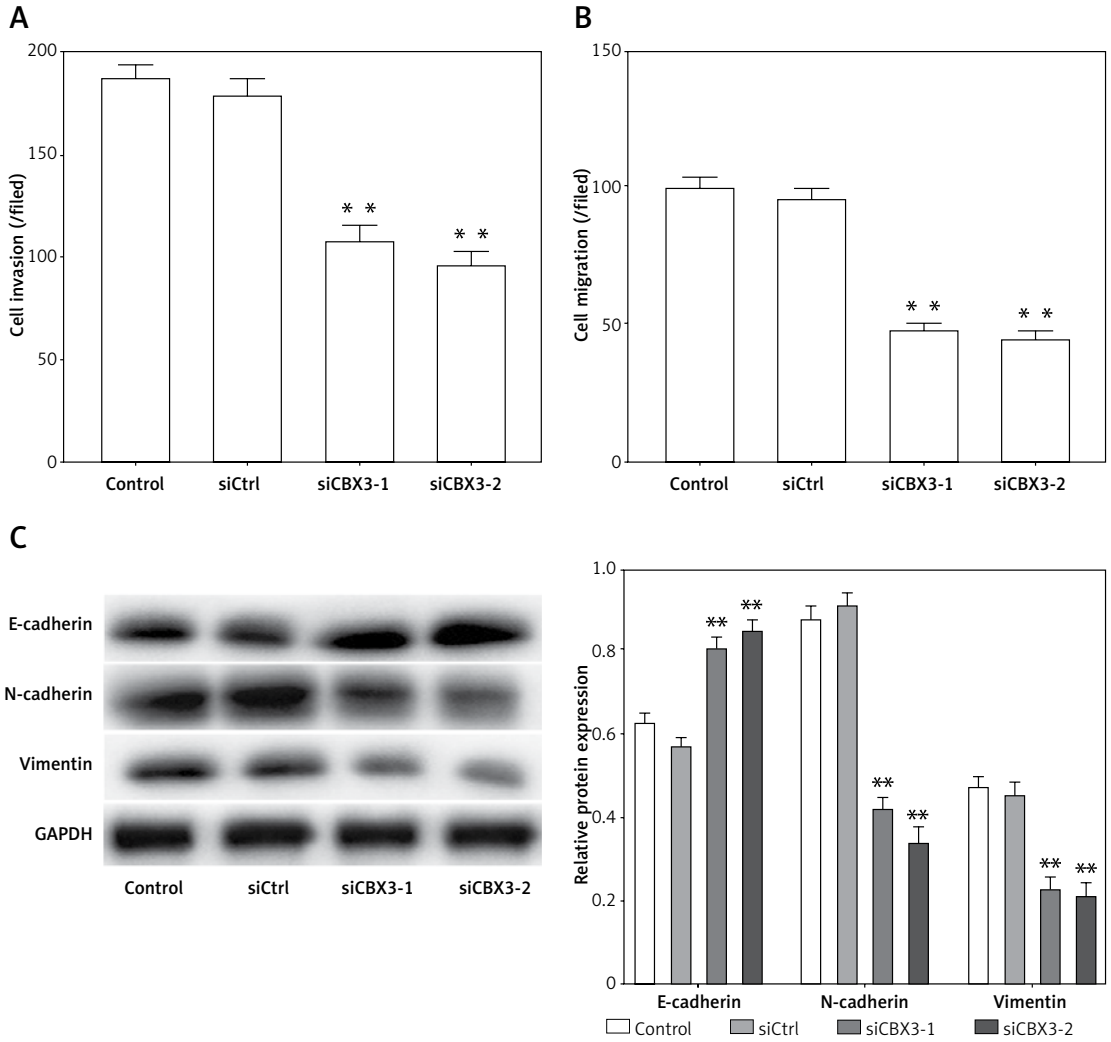

Figure 3. Knockdown of CBX3 suppressed OC cell invasion and migration. **A** – Cell invasion was detected by Transwell Matrigel invasion assays in SKOV3 cells after transfection with siCBX3s. **B** – Wound healing assay was applied to detected cell migration in SKOV3 cells after transfection with siCBX3s. **C** – Relative EMT related protein expression was detected by western blot assay; $^{**}p < 0.01$ vs. control group

These findings suggest that CBX3 is involved in the progression of ovarian cancer.

Alteration of glucose metabolism is considered as a hallmark of cancer. In cancer, aerobic glycolysis was activated, thereby promoting tumor growth and metastasis [9]. A recent report suggested that enhancing the expression of MIEF2 promoted ovarian cancer cellular functions through activating aerobic glycolysis [15]. In gastric cancer, the activated aerobic glycolysis was inhibited by H19 knockdown, thus inhibiting the malignant progression of tumors [16]. Similarly, the present study revealed that glycolysis was activated in OC cells. CBX3 knockdown inhibited cancer cellular function by suppressing glycolysis.

Furthermore, the results suggested that NCOR2 expression was increased by CBX3 knockdown and negatively associated with CBX3 expression, and similar results were also found in lung adenocarcinoma [17].

NCOR2 is a nuclear corepressor and is involved in the pathogenesis of cancer. Studies have shown that decreased NCOR2 facilitated tumor cell growth, invasion and migration in hepatocellular carcinoma [18]. In addition, elevated expression of NCOR2 inhibited cell growth and chemical resistance and promoted cell apoptosis in head and neck squamous cell carcinoma [19]. Only one study suggested that NCOR2 was significantly associated with ovarian cancer with microarray analysis [20]. However, the role and function of NCOR2 in ovarian cancer are still vague.

We found that NCOR2 was down-regulated in both ovarian cancer tissues and cell lines and CBX3 promoted tumorigenesis by inhibiting expression of NCOR2 in ovarian cancer. Moreover, NOCR2 has been identified as a vital factor in the maintenance of metabolic signaling [21], suggesting that NCOR2 may be associated with glycolysis

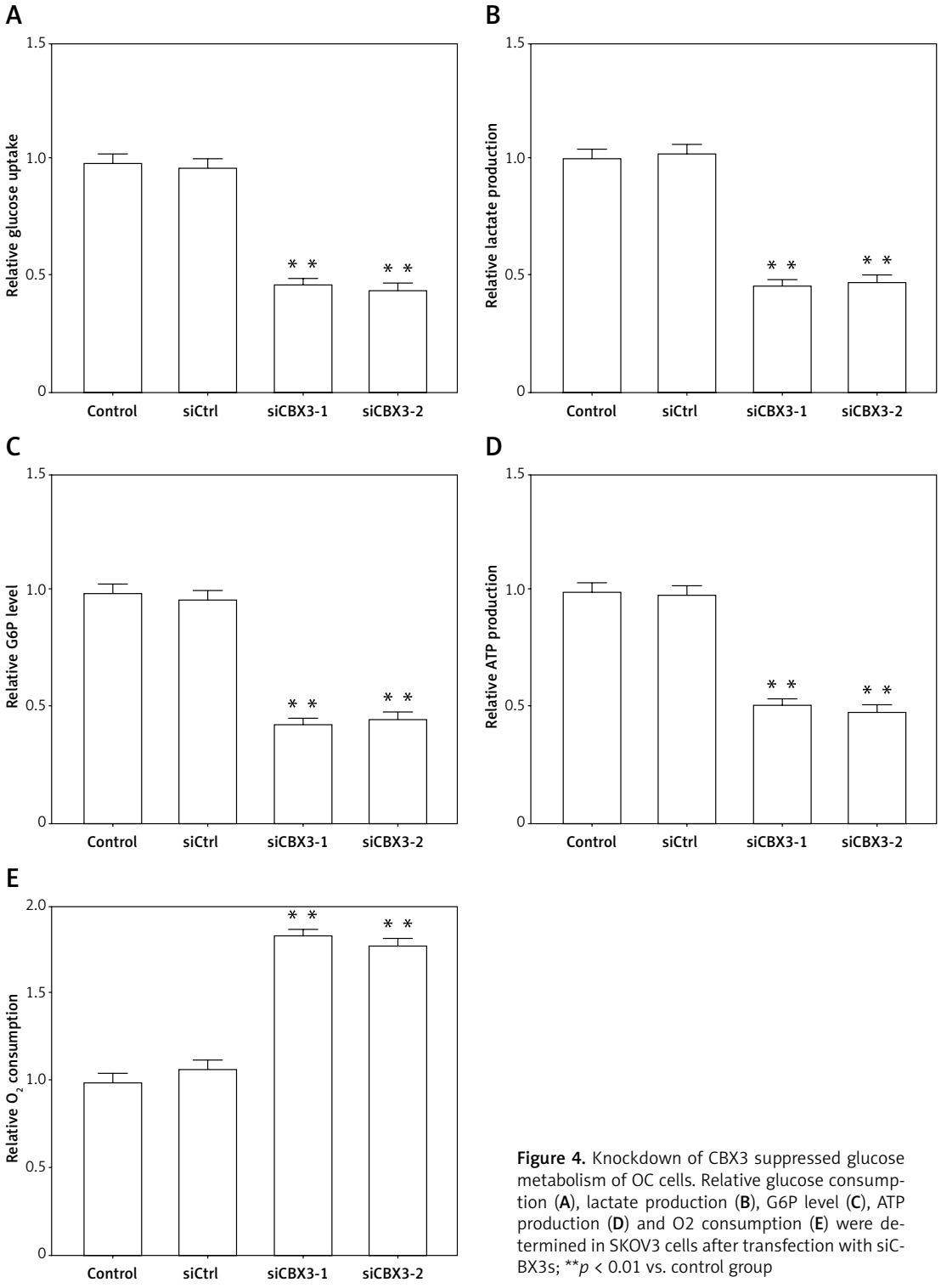

Figure 4. Knockdown of CBX3 suppressed glucose metabolism of OC cells. Relative glucose consumption (**A**), lactate production (**B**), G6P level (**C**), ATP production (**D**) and O2 consumption (**E**) were determined in SKOV3 cells after transfection with siCBX3s; $^{**}p < 0.01$ vs. control group

in cancer. In this study, the glycolysis inhibited by CBX3 knockdown was reversed by inhibition of NCOR2.

P53, an important tumor suppressor gene, is involved in the regulation of a variety of cellular processes in cancer progression, including the cell cycle, DNA repair, apoptosis and autophagy [22]. p21 is a downstream target of p53, which binds to the p21 promoter and activates its transcription [23]. In addition, p21 is a negative regulator of the cell cycle, and regulates various physiological activities, such as cell growth, differentiation, DNA repair, and senescence [24]. An increasing number of studies have demonstrated that inhibition of the p53/p21 pathway promoted cell proliferation and tumorigenesis, and suppressed

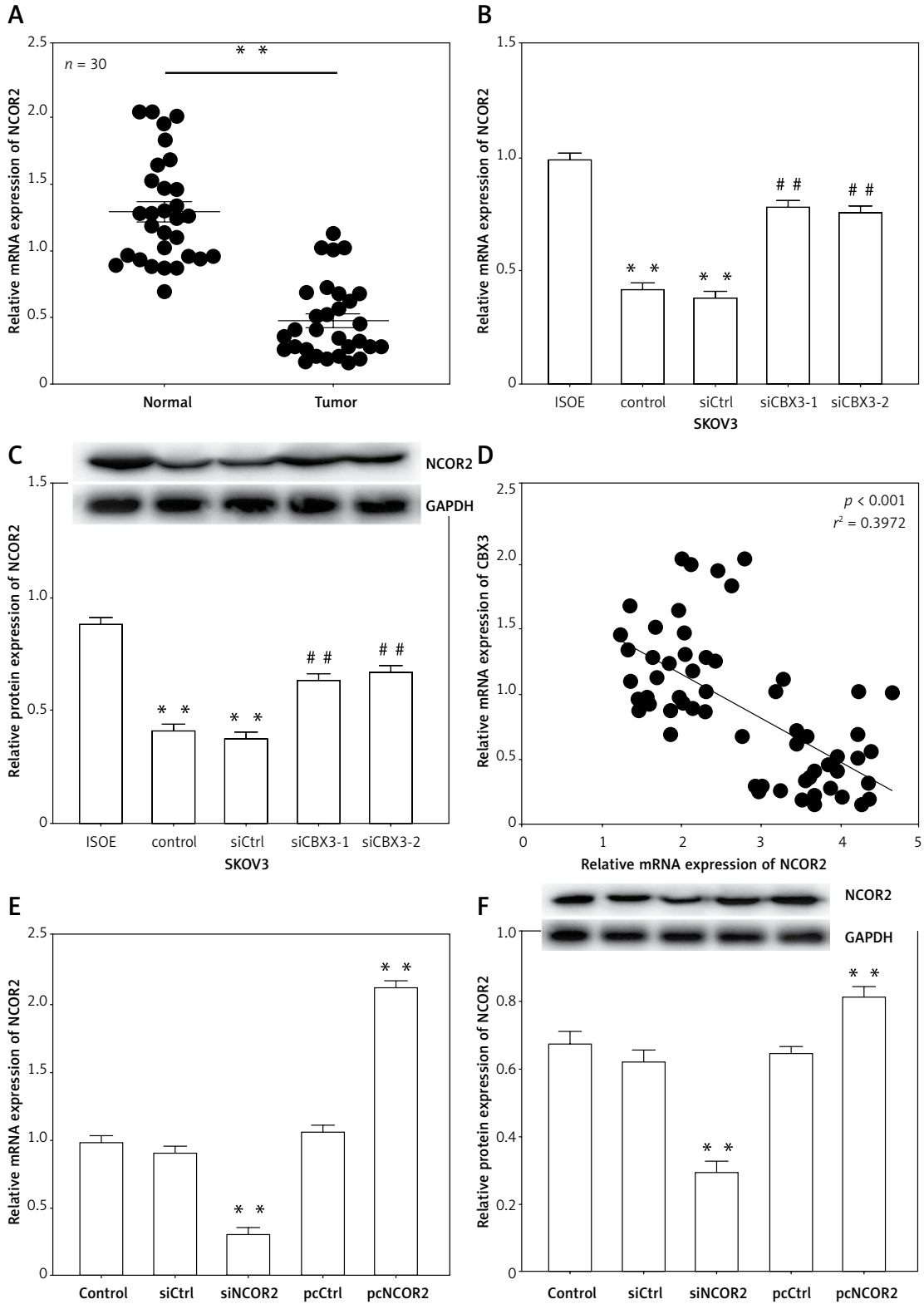

Figure 5. CBX3 blocked the activation of p53/p21 pathway by inhibiting NCOR2. **A** – The mRNA expression of NCOR2 was evaluated by qRT-PCR in OC tumor and adjacent non-tumor tissues from 30 OC patients; $^{**}p < 0.01$ vs. normal group. Relative mRNA (**B**) and protein (**C**) expression of NCOR2 were detected by qRT-PCR and Western blot assays in OC cell lines after transfection with siCBX3s; $^{**}p < 0.01$ vs. ISOE cell line, $^{\#\#}p < 0.01$ vs. siCtrl group. **D** – Correlation analysis between CBX3 and NCOR2 relative mRNA expression in OC tissues. Relative mRNA (**E**) and protein (**F**) expression of NCOR2 were confirmed by qRT-PCR and western blot assays after transfection with siNCOR2 and pcNCOR2; $^{**}p < 0.01$ vs. control group

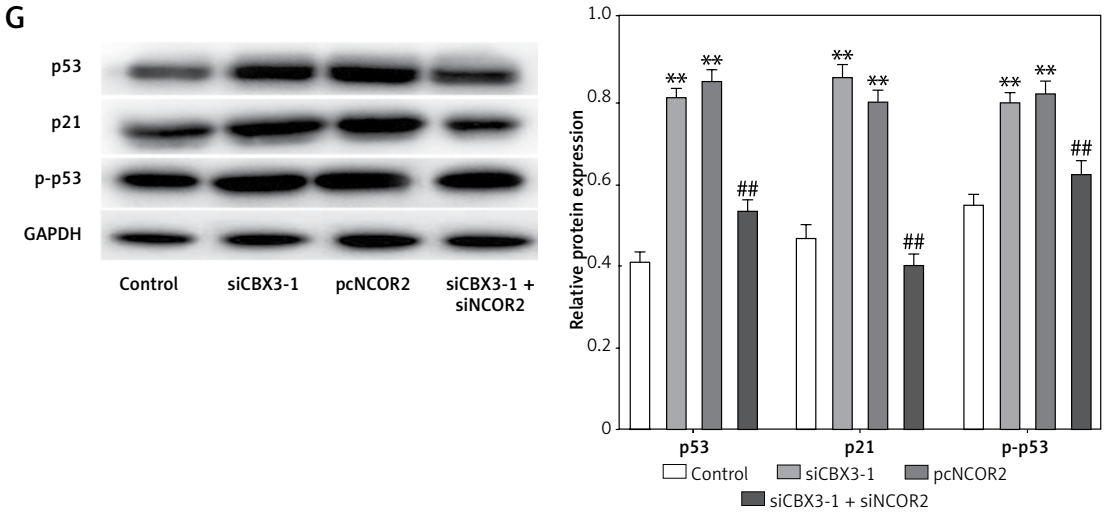

Figure 5. Cont. **G** – Relative protein expression of p53, p21 and p-p53 were detected by western blot assay; $^{**}p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. siCBX3-1 group

cell senescence in colorectal cancer [25, 26]. The p53/p21 signaling pathway was also involved in regulating the proliferation, migration and invasion of pancreatic ductal adenocarcinoma and endometrial cancer cells [27, 28]. In ovarian cancer, USP39 knockdown-induced cell cycle arrest was also found to be associated with the p53/p21 signaling pathway [29]. In this study, the results revealed that CBX3 regulated ovarian cancer progression by inactivating the p53/p21 pathway via inhibiting NCOR2. Emerging studies have also demonstrated that p53/p21 acts as a tumor suppressor by regulating the Warburg effect. The activated reprograming of glucose metabolism in a p53-dependent manner facilitated human colon adenocarcinoma cell growth [30]. Additionally, p21, targeted by miR-512-5p, mediates non-small cell lung cancer cell apoptosis and glycolysis [31]. Consistently, the inactivation of p53/p21 promoted the glycolysis inhibited by CBX3 knockdown and NCOR2 overexpression.

In the present study, we explored the role and molecular mechanism of CBX3 in OC, as well as its regulatory effect on glycolysis in OC. These findings provide a novel and potential biomarker for the treatment of ovarian cancer. Furthermore, blocking glycolysis may represent a new possibility for the treatment of OC; however, the specific mechanism of the glycolysis in OC is currently limited. The study found that CBX3 regulates glycolysis in ovarian cancer, but more *in vivo* and clinical research is required to further explore the specific mechanism underlying these effects.

In conclusions, CBX3 was highly expressed in OC tissues and cell lines and was negatively correlated with NCOR2. Furthermore, CBX3 promoted the viability, migration, and invasion of OC cells through activated p53/p21-mediated glycolysis via inhibiting NCOR2. Therefore, CBX3 may be a novel target for the prognosis and treatment of OC.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

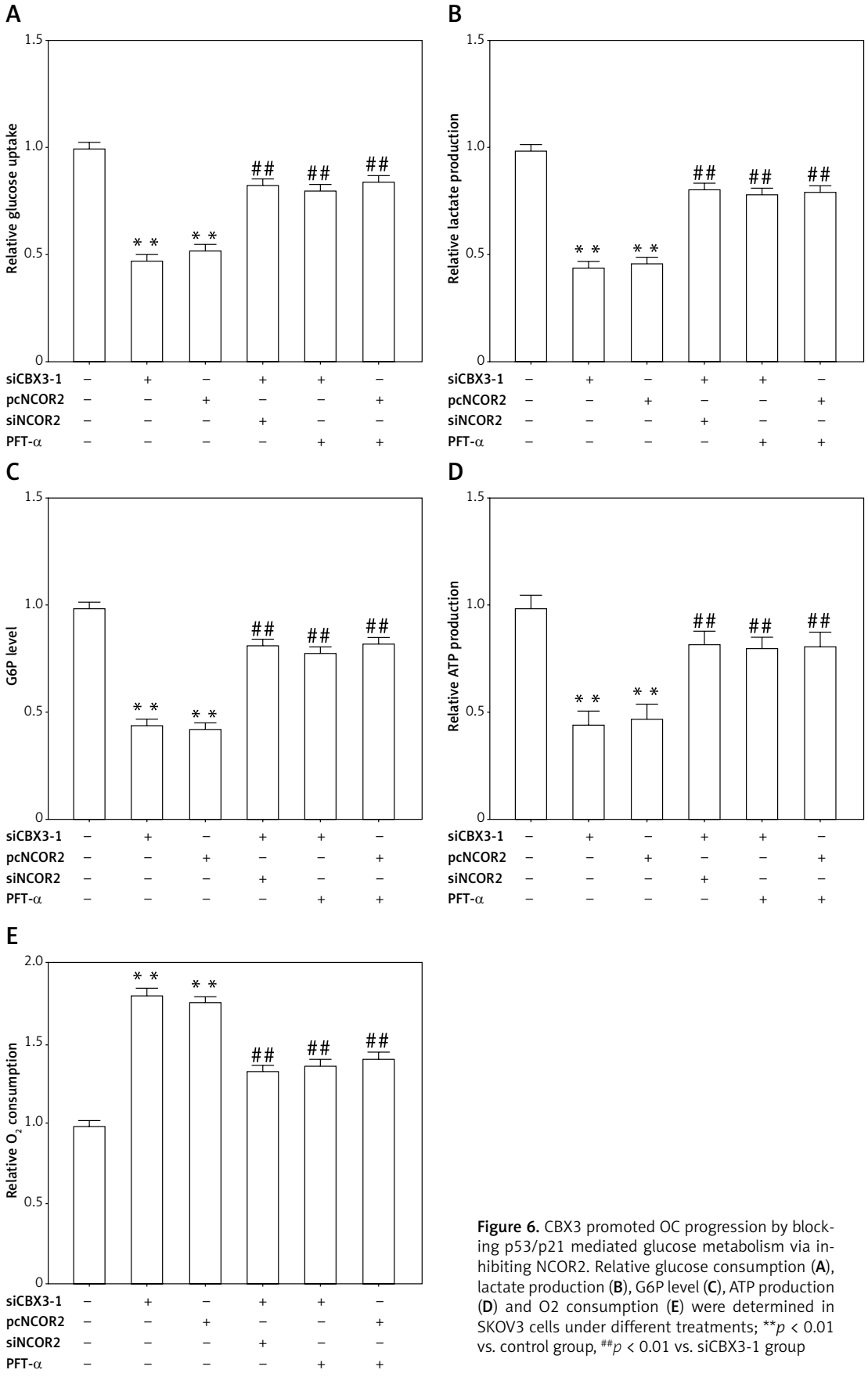

Figure 6. CBX3 promoted OC progression by blocking p53/p21 mediated glucose metabolism via inhibiting NCOR2. Relative glucose consumption (**A**), lactate production (**B**), G6P level (**C**), ATP production (**D**) and O2 consumption (**E**) were determined in SKOV3 cells under different treatments; $^{**}p < 0.01$ vs. control group, $^{\#\#}p < 0.01$ vs. siCBX3-1 group

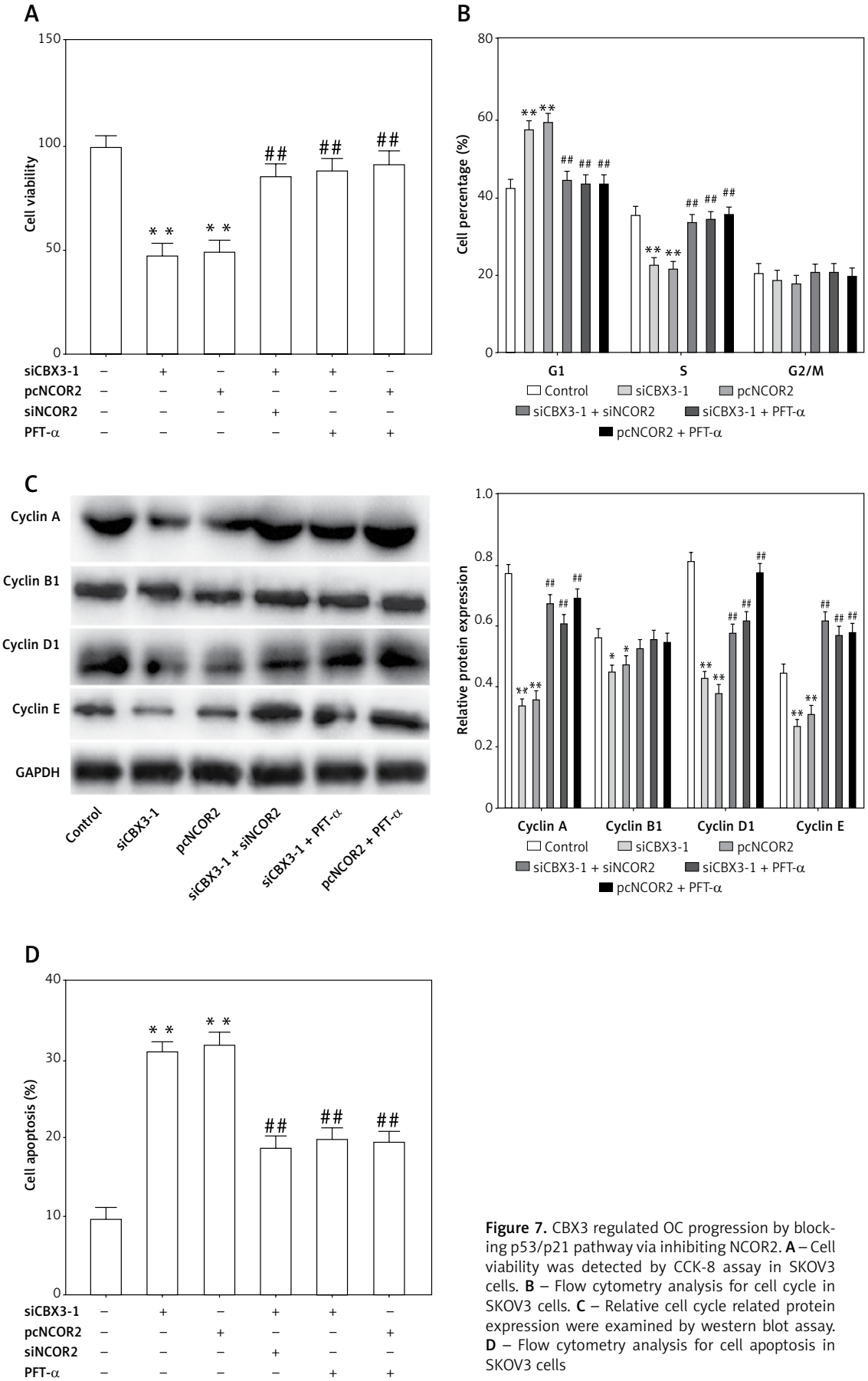

Figure 7. CBX3 regulated OC progression by blocking p53/p21 pathway via inhibiting NCOR2. **A** – Cell viability was detected by CCK-8 assay in SKOV3 cells. **B** – Flow cytometry analysis for cell cycle in SKOV3 cells. **C** – Relative cell cycle related protein expression were examined by western blot assay. **D** – Flow cytometry analysis for cell apoptosis in SKOV3 cells

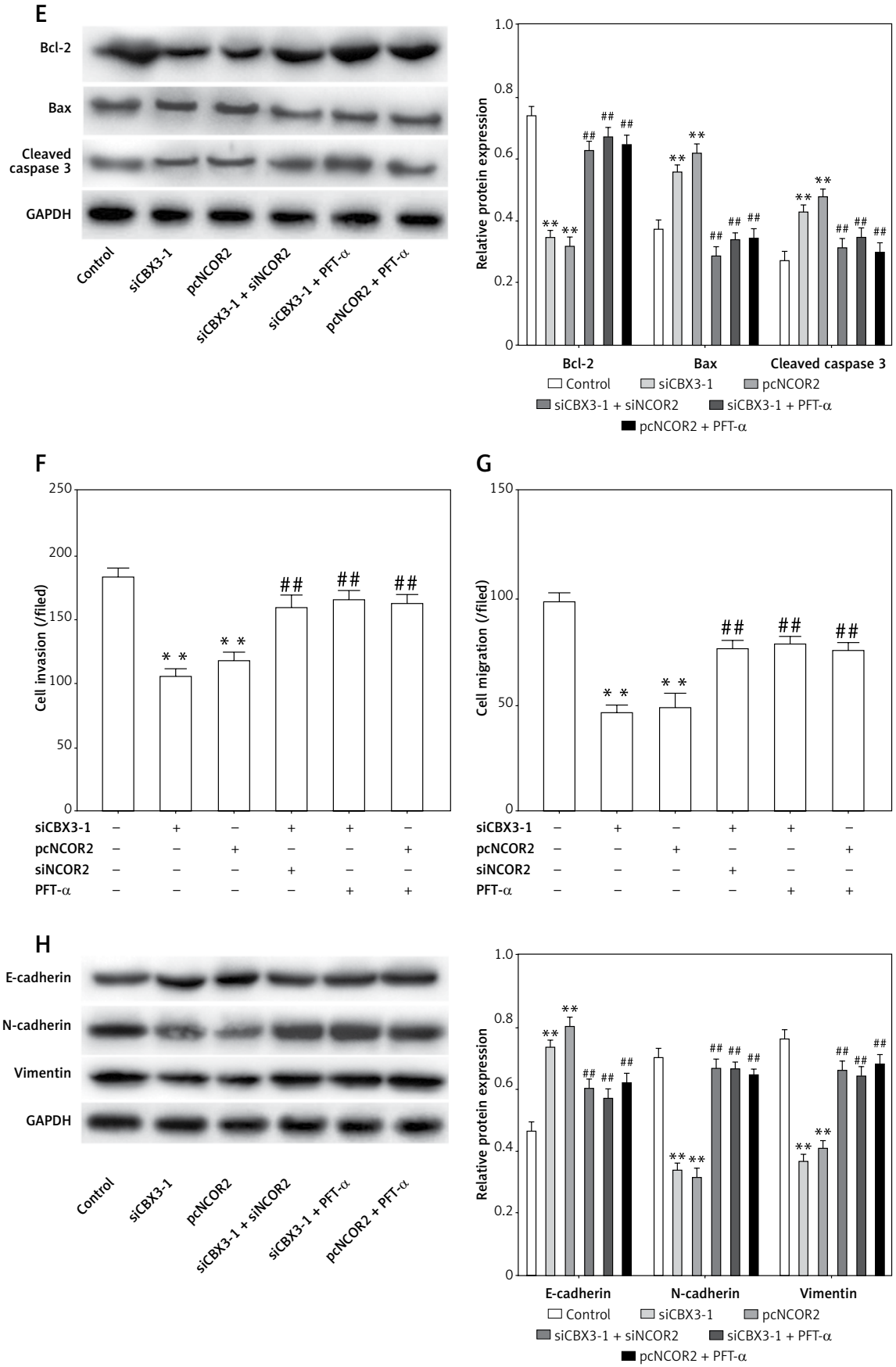

Figure 7. Cont. **E** – Relative cell apoptosis related protein expression were detected by western blot assay. **F** – Cell invasion was detected by Transwell Matrigel invasion assays in SKOV3 cells. **G** – Wound healing assay was applied to detected cell migration in SKOV3 cells. **H** – Relative EMT related protein expression were detected by western blot assay; $^{**}p < 0.01$ vs. control group; $^{\#\#}p < 0.01$ vs. siCBX3-1 group

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