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Silencing of NADPH Oxidase 4 Attenuates Hypoxia Resistance in Neuroblastoma Cells SH-SY5Y by Inhibiting PI3K/Akt-Dependent Glycolysis

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Hypoxia-induced chemoresistance is a major obstacle in the development of effective cancer therapy. In our study, the reversal abilities of NADPH oxidase 4 (NOX4) silence on hypoxia resistance and the potential mechanism were investigated. Our data showed that the expression of NOX4 was upregulated in human neuroblastoma cells SH-SY5Y under hypoxia condition time dependently. Knockdown of NOX4 expression by siRNA inhibited glycolysis induced by hypoxia through decreasing the expression of glycolysis-related proteins (HIF-1 , LDHA, and PDK1), decreasing glucose uptake, lactate production, and ROS production, while increasing mitochondria membrane potential. Moreover, NOX4 silence inhibited cell growth under hypoxia condition through suppressing cell proliferation and proliferation-related proteins (Ki-67 and PCNA) compared with the hypoxia 24 h + siRNA NC group. Further, Western blot experiments exhibited that NOX4 siRNA could downregulate the rate of p-Akt/Akt. Treatment with PI3K/Akt signaling activator IGF-1 blocked, while treatment with Akt inhibitor perifosine enhanced the inhibitory effect of si-NOX4 on glycolysis and cell growth. In summary, knockdown of NOX4 had the ability of reversing hypoxia resistance, and the major mechanism is considered to be the inhibition of glycolysis and cell growth via the PI3K/Akt signaling pathway. Therefore, NOX4 could be a novel target against hypoxia resistance in neuroblastoma.

Key words: NADPH oxidase 4 (NOX4); Hypoxia resistance; Neuroblastoma; Glycolysis; PI3K/Akt

INTRODUCTION

Neuroblastoma is the most common solid tumor in children, responsible for 15% of all childhood cancer deaths[1]. Aberrant proliferation of undifferentiated neural crest cell progenitors in the developing sympathoadrenal lineage of the nervous system contributed to the carcinogenesis of neuroblastoma[2]. Although treatment, including surgery, chemotherapy, and immunotherapy, has improved against neuroblastoma, the 3-year disease-free survival rate is still only about 60% for metastatic disease[3,4]. Therefore, it is urgent to explore new and more effective targets against neuroblastoma progression.

Hypoxia, the main cause of treatment failure in various types of malignancies, is frequently observed in the center of solid tumors[5,6]. Accumulating evidence demonstrated that a hypoxic microenvironment is coincident with the development and maintenance of cancers[7]. In the hypoxic environment, cancer cells gain hypoxia resistance through undergoing genetic and adaptive changes to survive and proliferate[8]. Hypoxic cells are considered to be resistant to most anticancer drugs[9]. Hypoxia can induce hypoxia-inducible factor 1 (HIF-1), which is an oxygen-dependent transcriptional activator and plays crucial roles in the angiogenesis of tumors and mammalian development[10]. The expression of HIF-1 can increase the viability of hypoxic cells[11]. Moreover, hypoxia is also reported to induce a high rate of glycolysis in tumors[12]. The "Warburg effect" or "aerobic glycolysis" refers to the phenomenon that cancer cells undergo an aberrant metabolic shift to glycolytic energy dependence in the presence of oxygen[13]. Therefore, suppressing glycolysis and viability of cancer cells in a hypoxic environment can help to attenuate hypoxia resistance of tumor cells.

NADPH oxidases are a family of enzymes that can generate superoxide or hydrogen peroxide. NADPH oxidase 4 (NOX4), which is a member of NADPH oxidases, has been found to be deregulated in various cancers and involved in cancer proliferation and metastasis. Zhang et al. reported that NOX4 promoted cell proliferation and metastasis in non-small cell lung cancer cells through

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regulation of the PI3K/Akt pathway[14]. Silencing NOX4 inhibited cell invasion of gastric cancer cells through the JAK2/STAT3 signaling pathway[15]. Suppression of NOX attenuated hypoxia-induced dysfunction of endothelial progenitor cells[16]. Thus, we hypothesized that silencing NOX4 may inhibit hypoxia resistance of tumor cells.

In our present study, we found that NOX4 was highly expressed in human neuroblastoma SH-SY5Y cells that underwent hypoxia treatment. Silencing NOX4 suppressed glycolysis induced by hypoxia and cell growth through inhibiting the PI3K/Akt signaling pathway. Silencing NOX4 attenuated hypoxia resistance, which might suppress tumor progression and drug resistance in human neuroblastoma.

MATERIALS AND METHODS

Cell Culture and Treatment

The human neuroblastoma cell line SH-SY5Y was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Science, Logan, UT, USA) in a humidified atmosphere of 5% CO_2 at 37°C. A hypoxic culture condition was created by incubating cells in a sealed modular incubator chamber (Billups Rothenberg, Del Mar, CA, USA) flushed with 5% CO_2 and 94% N_2. Because the culture flasks contained ambient oxygen at the beginning of the experiments, the final oxygen content in the hypoxia chamber was 1.0% after achieving air equilibrium[9]. After culturing in the hypoxia environment for different times (2, 8, and 24 h), cells were collected for the following experiments. PI3K/Akt signaling activator IGF-1 and Akt inhibitor perifosine were obtained from PeproTech China (Suzhou, P.R. China) and used to treat cells at concentrations of 10 ng/ml and 10 μM, respectively.

Western Blot Analysis

Cells were collected and lysed in lysis buffer (Beyotime, Shanghai, P.R. China), and the concentrations of proteins were determined using a BCA protein assay kit (Beyotime). The same amount of proteins were separated by 10% SDS-PAGE gel and then transferred into PVDF membranes (Millipore, Boston, MA, USA). After blocking with 5% bovine serum albumin (BSA) for 1 h at room temperature, the membranes were incubated with primary antibodies obtained from Cell Signaling Technology [anti-NOX4, anti-HIF-1 , anti-LDHA, anti-PDK1, anti-Ki-67, anti-PCNA, anti-PI3K, anti-AKT, anti-p-AKT, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] at 4°C overnight. Then membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The protein signals were detected using the BeyoECL Plus Kit (Beyotime) according to the manufacturer's instruction.

Cell Transfection

Short interfering RNA (siRNA) against NOX4 and negative control (siRNA NC) were purchased from GenePharma (Shanghai, P.R. China). Cells were seeded into a 96-well plate to reach 60% confluence. Then cells were transfected with NOX4 siRNA or siRNA NC, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of Glucose Uptake Level

Glucose in the medium was detected by an Amplex Red Glucose/Glucose Oxidase Kit (Invitrogen, Eugene, OR, USA) using a standard curve prepared with serial dilutions of DMEM (11 mmol/L glucose) into glucose-free McCoy's 5A medium. A microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to read the fluorescence. The glucose uptake level was then calculated.

Measurement of Lactate Generation

The Lactic Acid Assay Kit (KeyGEN Biotech, Nanjing, P.R. China) was used for measurement of lactate generation. The culture media supernatant was collected for lactate detection according to the manufacturer's instructions. The absorbance was determined by a microplate spectrophotometer. The amount of lactate generation was calculated as follows: lactate generation (mM) = 3 $(OD_{sample} - OD_{blank})/(OD_{standard} - OD_{blank})$.

Assay for ROS and Mitochondrial Membrane Potential ($\Delta\Psi m$)

Cells from each treatment were collected and washed by PBS twice, and then were resuspended in 500 μl of dichloro-dihydro-fluorescein diacetate (DCFH-DA) (10 μM) for ROS and $DiOC_6$ (1 μmol/L) for the level of m in the dark for 30 min at 37°C. Then all samples were detected through flow cytometry as previously described[17].

Cell Growth Assay

Different groups of cells were seeded at 5 10^3 cells per well into 96-well plates. At indicated time points, 10 μl of cell counting kit 8 (CCK-8) solution (Beyotime) was added to the cultures and incubated at 37°C for 2 h. The absorbance was measured at 450 nm using a microplate spectrophotometer.

Statistical Analysis

All data were reported as means ± standard deviation (SD) from three independent studies. Group comparison was performed using Student's *t*-test with SPSS 19.0

software (SPSS, Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered as statistically significant.

RESULTS

NOX4 Is Overexpressed in SH-SY5Y Cells Under Hypoxia Condition

We first performed Western blot analyses to determine NOX4 expression in human neuroblastoma SH-SY5Y cells under hypoxia condition. The results revealed that hypoxia increased NOX4 expression in SH-SY5Y cells in a time-dependent manner compared with the normoxia group ($p < 0.05$, $p < 0.01$) (Fig. 1A and B). SH-SY5Y cells were transfected with NOX4 siRNA to decrease the relative expression of NOX4 for our following experiments and were detected through Western blot ($p < 0.05$) (Fig. 1C and D). These results indicated that NOX4 is overexpressed in SH-SY5Y cells under hypoxia condition.

Knockdown of NOX4 Inhibits Glycolysis Induced by Hypoxia

Hypoxia is reported to induce a high rate of glycolysis in tumors. Thus, we detected the protein level of glycolysis-related proteins through Western blot. Our data showed that after being under hypoxia condition for 24 h, the expressions of HIF-1 , LDHA, and PDK1 were all remarkably upregulated in SH-SY5Y cells compared with the normoxia group. However, knockdown of NOX4 by transfection with NOX4 siRNA significantly decreased the expression of these proteins mentioned above compared with the hypoxia 24 h + siRNA NC group ($p < 0.01$, $p < 0.05$) (Fig. 2A and B). Moreover, hypoxia induced glycolysis with increased glucose uptake, lactate production, and ROS production, and decreased mitochondria membrane potential. NOX4 siRNA counteracted the promoting effects of hypoxia on glycolysis through downregulating glucose uptake, lactate production, and ROS production while upregulating mitochondria membrane potential compared with the hypoxia 24 h + siRNA NC group ($p < 0.05$, $p < 0.05$) (Fig. 2C–F). Our data suggested that knockdown of NOX4 inhibited glycolysis induced by hypoxia.

Knockdown of NOX4 Suppresses Cell Growth Under Hypoxia Condition

We then explored the effect of NOX4 on cell growth. Hypoxia increased the cell proliferation rate and the expression of Ki-67 and PCNA compared with the normoxia group. However, NOX4 knockdown downregulated the high cell proliferation rate and the expression of Ki-67 and PCNA significantly compared with the hypoxia 24 h + siRNA NC group ($p < 0.05$, $p < 0.01$, $p < 0.05$) (Fig. 3A–C). Our results demonstrated that NOX4 siRNA inhibited cell growth of SH-SY5Y cells under hypoxia condition.

Knockdown of NOX4 Suppresses Glycolysis and Cell Growth Through Inhibiting the PI3K/Akt Pathway

The PI3K/Akt pathway is a well-documented downstream signaling pathway of NOX[18]. Therefore, we sought to investigate whether NOX4 attenuated hypoxia resistance through the PI3K/Akt pathway. As shown in Figure 4,

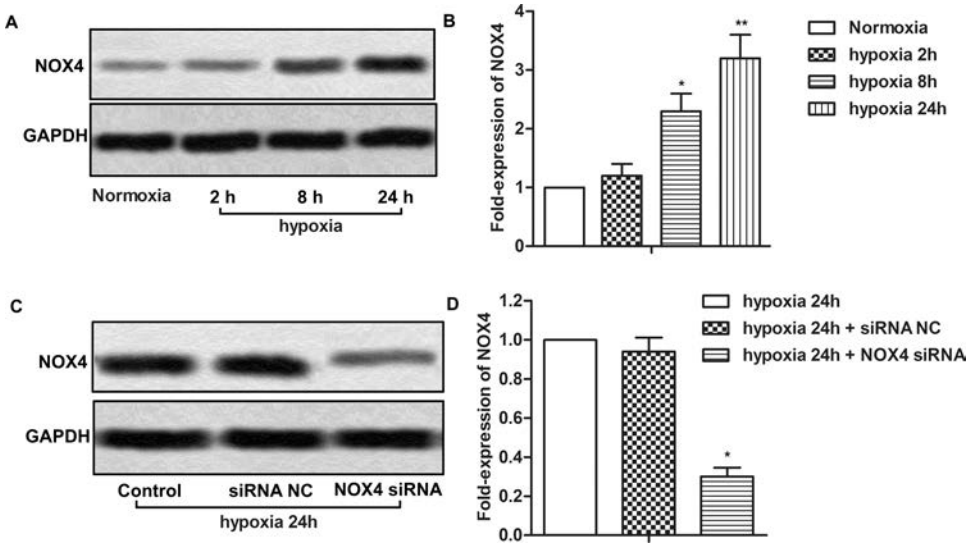

Figure 1. NADPH oxidase 4 (NOX4) is overexpressed in SH-SY5Y cells under hypoxia condition. SH-SY5Y cells transfected with NOX4 short interfering RNA (siRNA) or siRNA NC were cultured in hypoxia condition for different times. (A–D) Relative expression of NOX4 was detected through Western blot. All data were represented as the mean ± standard deviation (SD) from three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with the normoxia group or the hypoxia 24 h + siRNA NC group, respectively.

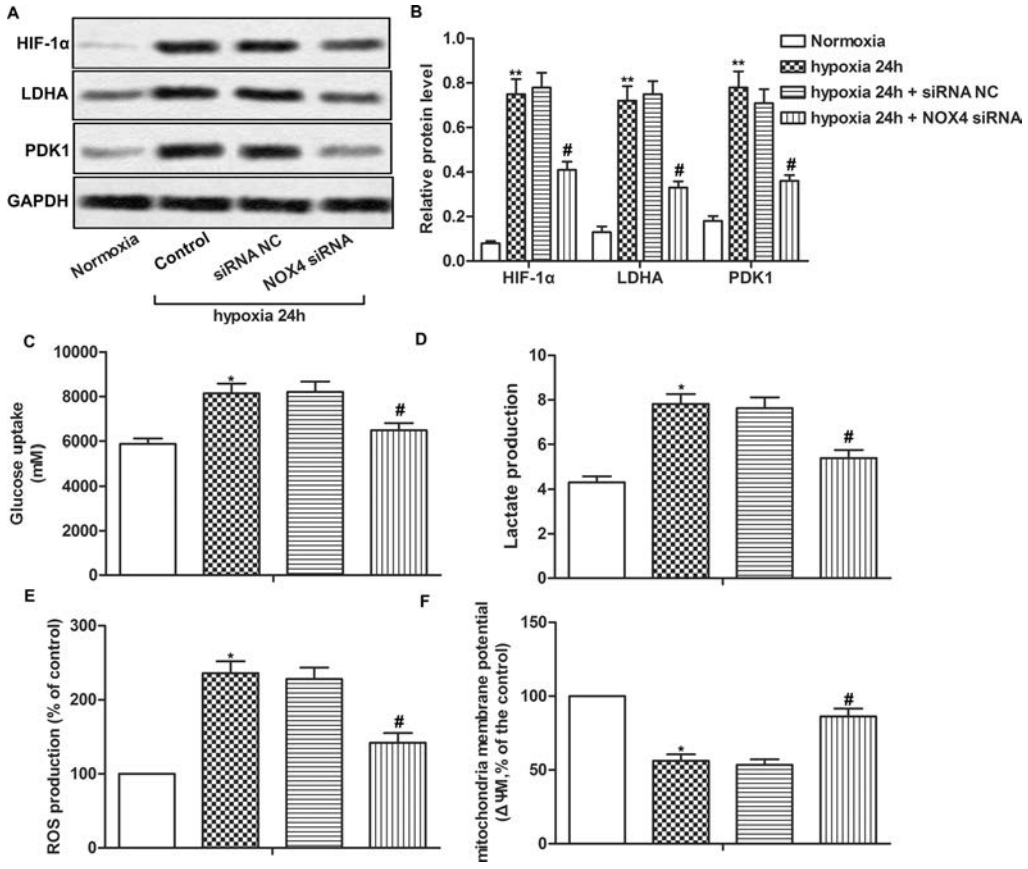

Figure 2. Knockdown of NOX4 inhibits glycolysis induced by hypoxia. SH-SY5Y cells transfected with NOX4 siRNA or siRNA NC were cultured in hypoxia condition for 24 h. (A, B) Relative expression of hypoxia-inducible factor 1 (HIF-1), LDHA, and PDK1 was detected through Western blot. (C) Glucose uptake, (D) lactate production, (E) ROS production, and (F) mitochondria membrane potential were measured as described in Materials and Methods. All data are represented as the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with the normoxia group, #$p < 0.05$ compared with the hypoxia 24 h + siRNA NC group.

NOX4 siRNA remarkably reduced the high expression of PI3K and LDHA, the rate of p-Akt/Akt, and cell growth induced by hypoxia. Treatment of SHSY-5Y cells with PI3K/Akt signaling activator IGF-1 significantly blocked the inhibitory effect of si-NOX4 on glycolysis and proliferation. However, treatment with the Akt inhibitor perifosine enhanced the inhibitory effect of si-NOX4 on glycolysis and cell growth. Moreover, neither perifosine nor IGF-1 showed significant toxicity on SH-SY5Y cells ($p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.01$, $p < 0.05$) (Fig. 4E). These data indicated that NOX4 siRNA suppressed glycolysis and cell growth of SH-SY5Y cells through inhibiting the PI3K/Akt pathway.

DISCUSSION

Hypoxia is often found in solid tumors, especially in the center of rapidly growing cancers because of incomplete blood vessel networks[17]. Tumor cells not only survive under hypoxia condition but also increase metastasis ability and tolerance to anticancer therapy[18,19]. Hypoxia-induced drug resistance is one of the major obstacles of development of effective chemotherapy for cancer treatment. Therefore, attenuating hypoxia resistance can help to explore more effective therapies against tumor progression. In our present study, we found that knockdown of NOX4 suppressed hypoxia resistance in human neuroblastoma cells SH-SY5Y through inhibiting glycolysis induced by hypoxia and cell growth via the PI3K/Akt signaling pathway, indicating that NOX4 might be a novel target against neuroblastoma malignant progression.

NOX4, a member of the NADPH oxidases, has been identified to play an important role in the regulation of hypoxia-induced dysfunction. Liu et al. found that the expression of NOX (NOX2 and NOX4) was significantly upregulated in hypoxia-treated endothelial progenitor cells, and suppression of NOX attenuated hypoxia-induced dysfunction of endothelial progenitor cells[16]. Cycling hypoxia increased the expression of NOX4 and significantly promoted tumor invasion both in vitro

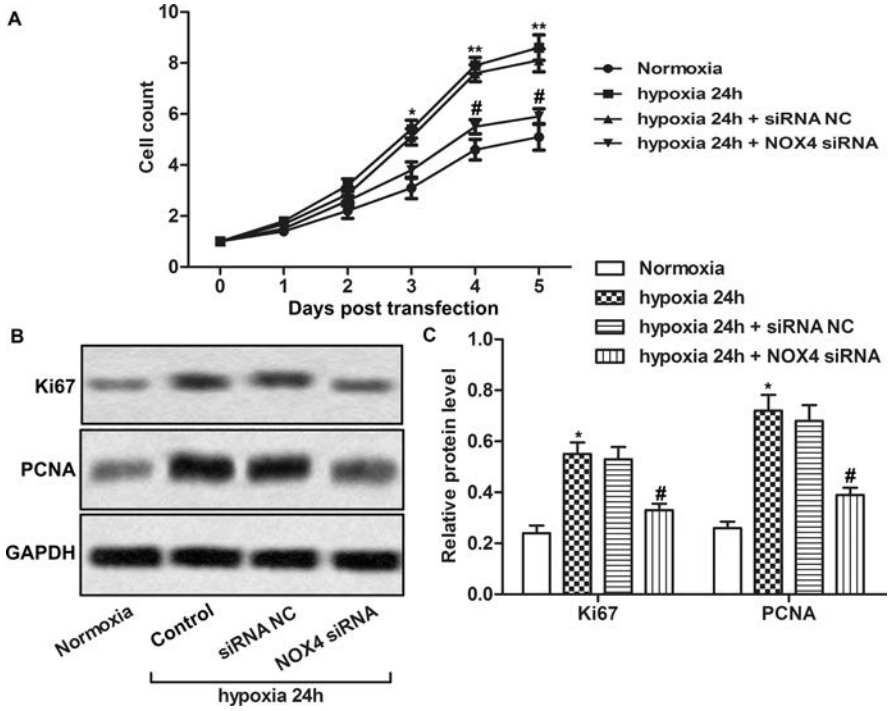

Figure 3. Knockdown of NOX4 suppresses cell growth under hypoxia condition. SH-SY5Y cells transfected with NOX4 siRNA or siRNA NC were cultured under hypoxic conditions for 24 h. (A) Cell growth was measured by cell counting kit 8 (CCK-8) assay. (B, C) Relative expression of Ki-67, PCNA was detected through Western blot. All data were represented as the mean ± SD from three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with the normoxia group, #$p < 0.05$ compared with the hypoxia 24 h + siRNA NC group.

and in vivo. However, NOX4 knockdown inhibited this effect[20]. In agreement with these previous studies, we also observed that relative expression of NOX4 was significantly upregulated in hypoxia-treated SH-SY5Y cells, and the longer the treated time, the higher the expression of NOX4. In order to investigate the role of NOX4 in the hypoxia resistance of neuroblastoma cells, NOX4 siRNA was transfected into SH-SY5Y cells to decrease the expression of NOX4 in our following experiments.

It has been well demonstrated that cancer cells undergo a metabolic switch from oxidative phosphorylation to glycolysis, which is important for the survival and proliferation of cancer cells in a hypoxic environment[21,22]. There are a great number of proteins including HIF-1 , LDHA, and PDK1 involved in the hypoxia-induced glycolysis. HIF-1 is induced in the hypoxia environment and transactivates more than 60 genes involved in angiogenesis, invasion, energy metabolism, tumor growth, and poor prognosis[23]. LDHA is a glycolytic enzyme that plays a crucial role in controlling the speed of glycolysis and maintaining the continuity of aerobic glycolysis in cancer cells[24,25]. PDK1 inactivates the pyruvate dehydrogenase enzyme complex that converts pyruvate to acetyl-coenzyme A, thereby inhibiting pyruvate oxidation via the tricarboxylic acid cycle to generate energy[26]. In our study, hypoxia increased the expression of glycolysis-related proteins, indicating that hypoxia induced glycolysis in SH-SY5Y cells. Previous studies elucidated that hypoxia led to a marked increase in the glucose uptake and production of lactate[27,28]. ROS are increased by hypoxia in various types of cells, and elevated ROS can cause collapse of the mitochondrial membrane potential[29–31]. Similarly, we found that hypoxia upregulated glucose uptake as well as lactate production. Besides that, ROS production was increased while mitochondria membrane potential was remarkably decreased by hypoxia. However, transfection of SH-SY5Y cells with NOX4 siRNA abolished the promoting role of hypoxia on glycolysis by decreasing glucose uptake, lactate production, and ROS production while increasing the mitochondria membrane potential compared with the hypoxia 24 h + siRNA NC group. Results above suggested that knockdown of NOX4 suppressed glycolysis induced by hypoxia in SH-SY5Y cells.

Hypoxia is reported to promote cell proliferation of various types of cancer cells. Hypoxia promoted tumor cell proliferation and migration through inducing miR-214 expression in gastric carcinoma cells[32]. Hypoxia also induced cell proliferation, invasion, and epithelial–mesenchymal transition (EMT) in human osteosarcoma

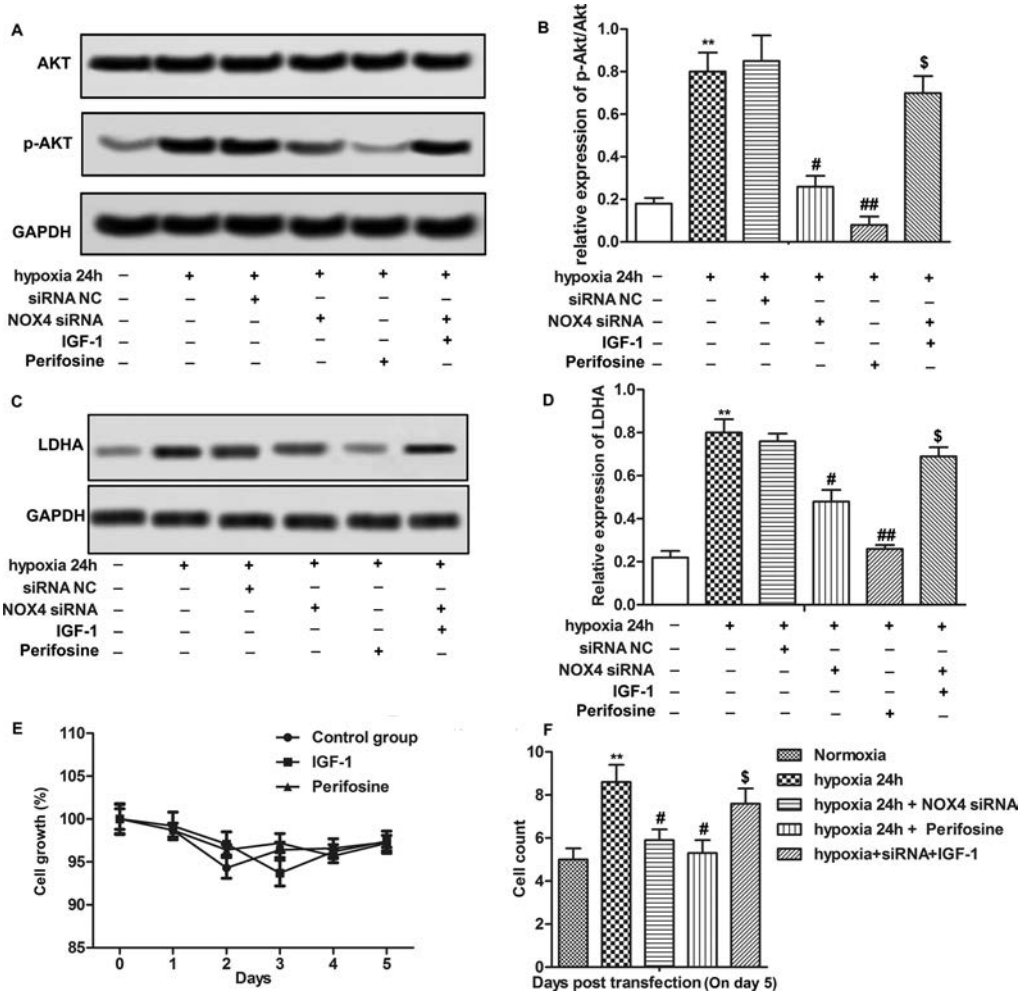

Figure 4. Knockdown of NOX4 suppresses glycolysis and cell growth through inhibiting the PI3K/Akt pathway. SH-SY5Y cells transfected with NOX4 siRNA were cultured in hypoxia condition for 24 h and received IFG-1 treatment or not. (A–D) Relative expression of PI3K, Akt, p-Akt, and LDHA was detected through Western blot. (E, F) Cell growth was measured by CCK-8 assay. All data were represented as the mean ± SD from three independent experiments. $^{**}p < 0.01$ compared with the normoxia group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the hypoxia 24 h + siRNA NC group, $\$p < 0.05$ compared with the hypoxia 24 h + NOX4 siRNA group.

cells[33]. Our data are in line with previous studies in that hypoxia promoted cell proliferation and increased the expression of proliferation-related proteins (Ki-67 and PCNA) in human neuroblastoma cells[32,33]. NOX4 siRNA counteracted the effects of hypoxia through suppressing cell proliferation and decreasing the expression of Ki-67 and PCNA, suggesting that knockdown of NOX4 inhibited cell growth under hypoxia condition.

Higher expression of PI3K/Akt was observed in hypoxia compared to normoxia[34]. Thus, we measured the expression of well-known effectors of the PI3K/Akt pathway including PI3K, AKT, and p-AKT. We found the increased expression of PI3K and increased rate of p-Akt/Akt under hypoxic conditions, while transfection with NOX4 siRNA significantly abolished these effects. Moreover, treatment with the PI3K/Akt signaling activator IGF-1 increased PI3K and p-AKT expression, stimulated the expression of LDHA, and promoted cell proliferation compared with the hypoxia 24 h + NOX4 siRNA group, while treatment with the Akt inhibitor perifosine had the opposite effects. Based on the results, we can say that NOX4 silencing attenuated hypoxia resistance through inhibiting glycolysis and cell growth via the PI3K/Akt signaling pathway in SH-SY5Y cells.

In conclusion, knockdown of NOX4 had the ability to reverse hypoxia resistance, and the major mechanism is considered to be the inhibition of glycolysis and cell growth via the PI3K/Akt signaling pathway. Therefore, NOX4 could be a novel target against hypoxia resistance in neuroblastoma.

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