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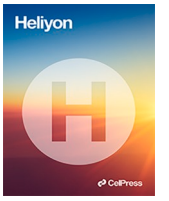

Research article

PAX6 enhances Nanog expression by inhibiting NOTCH signaling to promote malignant properties in small cell lung cancer cells

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ABSTRACT

Small cell lung cancer (SCLC) is a highly aggressive form of lung cancer. The aberrant regulation of Paired box 6 (PAX6) expression has been proposed to play an important oncogenic role in several cancer types. Nevertheless, there is limited knowledge regarding its function in SCLC. Here, we find that PAX6 overexpression promotes SCLC cell proliferation and cell cycle progression, while downregulation of PAX6 expression suppresses SCLC cell proliferation and cell cycle progression. Moreover, PAX6 enhances the enrichment of the ALDEFLUOR+ and CD133+ population and promotes sphere formation in SCLC cells. Additionally, upregulation or downregulation of PAX6 expression does not induce apoptosis in SCLC cells. Upregulation of PAX6 expression alleviates cisplatin or etoposide-induced apoptosis in SCLC cells, while downregulation of PAX6 expression aggravates cisplatin or etoposide-induced apoptosis in SCLC cells. Furthermore, PAX6 promotes the expression of stem cell factor Nanog. Interestingly, the downregulation of Nanog expression abolishes PAX6 promoted cell proliferation and cell cycle progression. Moreover, the inhibition of Nanog expression results in the inability of PAX6 to promote the increase of the ALDEFLUOR+ population, as well as the inability to mitigate apoptosis produced by cisplatin or etoposide in SCLC cells. Mechanically, PAX6 suppresses the activation of the NOTCH pathway to enhance Nanog expression. NOTCH pathway activation abolishes PAX6 promoted cell proliferation, cell cycle progression, ALDEFLUOR+ population enrichment, and apoptosis protection effect in SCLC cells. Our data indicates that PAX6 could be a critical factor for controlling cell proliferation, cell cycle progression, cancer stem cell properties, and apoptosis in SCLC.

1. Introduction

One of the most common malignancies in the world is lung cancer [1]. Small-cell lung cancer (SCLC) accounts for approximately 15 % of all lung malignancies, with a 5-year survival rate less than 5 % [2]. SCLC is an extremely aggressive disease that spreads quickly,

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metastasizes early, and develops resistance to available treatments [3]. While SCLC generally shows a high level of sensitivity to current chemotherapy regimens, the response to chemotherapy regimens is temporary, and chemorefractory SCLC almost always develops [3]. Unlike non-small cell lung cancer (NSCLC), which has a variety of targeted drugs, SCLC has not experienced significant advancement in treatments for a long time, and there is currently no authorized targeted therapies for SCLC [3]. Therefore, it is essential to investigate the mechanisms of chemoresistance in SCLC to identify potential treatment target capable of reversing chemoresistance.

The development of therapy resistance poses a significant challenge in the treatment of cancer patients, as cancer cells acquire mechanisms that neutralize the impact of therapeutic substances [4]. This results in the emergence of more aggressive clones, which ultimately contribute to a poor prognosis [4]. A subpopulation of tumor cells known as cancer stem cells (CSCs) have the ability to proliferate into several lineages and are innately resistant to anticancer therapies [5]. Currently, the treatment of tumors is a significant obstacle for oncologists due to the advanced stage of many cancers and the existence of resistant CSCs clones [6]. Hence, there is an urgent requirement for developing therapy that specifically targets CSCs in order to effectively prevent their potential recurrence and metastasis following treatment. Understanding the mechanism of CSC regulation in SCLC is essential to effectively address their resistance to therapy.

Paired-box 6 (PAX6), a PAX family member transcription factor encoded by a gene on human chromosome 11p13, is critical in embryonic development [7]. PAX6 also plays a crucial role as a transcription factor in regulating the development of the central nervous system [8]. Abnormal regulation of PAX6 expression leads to the occurrence of developmental abnormalities and the growth of malignancies [9]. PAX6 expression has been documented in multiple cancers, such as pancreatic cancer [10], lung adenocarcinoma [11], and glioblastoma [12]. PAX6 has been reported to regulate drug resistance [13], stem cell differentiation [14], cell invasiveness [15], and angiogenesis [16]. Nevertheless, the role of PAX6 in SCLC remains unknown, and the underlying mechanisms governing its roles in SCLC have not been elucidated. Recent studies indicate that Pax6 might also have role in the regulation of CSC properties. For example, PAX6 regulates GLI-SOX2 pathway in NSCLC to enhance the CSC properties [11]. Additionally, PAX6 might control epithelial-mesenchymal transition (EMT)-mediated CSC transformation [17]. Additional research is required to comprehend the function of PAX6 in SCLC.

In the current study, we explore the function of PAX6 in SCLC. Our study shows that PAX6 promotes SCLC cell proliferation and cell cycle progression. Importantly, PAX6 is critical for the enrichment of the CSC population, and for the enhancement of sphere formation in SCLC cells. Additionally, we find that PAX6 expression enhances the resistance to cisplatin or etoposide-induced apoptosis in SCLC cells. Suppression of PAX6 expression sensitizes SCLC cells to cisplatin or etoposide-induced apoptosis. Mechanically, we show that PAX6 suppresses the activation of the NOTCH pathway to enhance Nanog expression, thereby enhancing cells proliferation, cell cycle progression, CSC enrichment, and apoptosis protection effect in SCLC cells. Our study might provide a rationale for targeting PAX6 to inhibit CSC and overcome drug resistance in SCLC.

2. Materials and methods

2.1. Cell lines

The NCI-H446 and DMS-114 cell lines were acquired from the American Type Culture Collection (ATCC). NCI-H446 and DMS-114 cells were cultivated using RPMI-1640 medium supplemented with 10 % fetal bovine serum. The cells were cultured at a temperature of 37 °C in an atmosphere with 5 % CO2 and high humidity.

2.2. Reagents

Antibodies against Caspase-3, Caspase-8, Caspase-9, PARP, Nanog, NICD, c-Myc, HEY1, Flag, PAX6, and GAPDH were purchased from Cell Signaling Technology.

2.3. Western blot analysis

Whole-cell lysates were generated using RIPA buffer. The proteins were separated using SDS/PAGE, followed by blotting onto nitrocellulose membranes and subsequent probing with the corresponding primary antibodies. The secondary antibodies were conjugated with horseradish peroxidase (HRP). The chemiluminescent signals were detected using the Pierce ECL kit. The raw images of gels and blots were provided (Raw images in Supplementary Material).

2.4. ALDEFLUOR analysis

Cells were incubated with the ALDEFLUOR solution (ALDEFLUOR kit, Stem Cell Technologies) for 45 min at 37 °C, either in the presence or absence of N,N-diethylaminobenzaldehyde (DEAB). Flow cytometry was used to examine the cells.

2.5. Sphere culture

Six-well ultra-low attachment plates were seeded with single cells (500 cells/ml). In spherical culture medium (DMEM/F12 supplemented with B27, 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor, and 4 μg/ml heparin) [18], cells were

cultivated for five to seven days. The images of the spheres were taken, then after analysis by Image Pro Plus 6.0.

2.6. Plasmids

The shRNA against PAX6 or Nanog were designed by using InvivoGen's siRNA Wizard and cloned into pLKO.1-puro (Addgene). The shRNA target sequences of PAX6 were as follows: #1, 5′-GGTCTGTACCAACGATAACAT; #2, 5′-GTCTGTACCAACGATAACATA; #3, 5′-GACGGCATGTATGATAAACTA. The shRNA target sequence of Nanog was 5′-AACCAGACCTGGAACAATTCA. The control shRNA target sequence was 5′-CCTAAGGTTAAGTCGCCCTCG.

The plasmid used for overexpressing interested genes were constructed by using pLVX-IRES-Puro vector. Flag tag was added to the C-terminus of PAX6 coding sequence to express PAX6-Flag protein. HA tag was added to the C-terminus of NICD coding sequence to express NICD-HA protein.

2.7. Lentivirus preparation

10 % FBS was added to DMEM growth media used to cultivate HEK-293T cells. The cells were transfected at 70 % confluency. Using Lipofectamine 3000, cells were transfected with a DNA combination (Transfer Vector: psPAX2: pMD2.G = 4:3:1). To ensure that the lipofectamine-DNA complex was distributed equally, the cell culture plate was gently shaken. The media was changed out for new growth medium after the cells had grown for 24 h. After 72 h of transfection, virus-containing supernatant was collected, put onto a 20 ml syringe, and passed through a 0.45 μm filter.

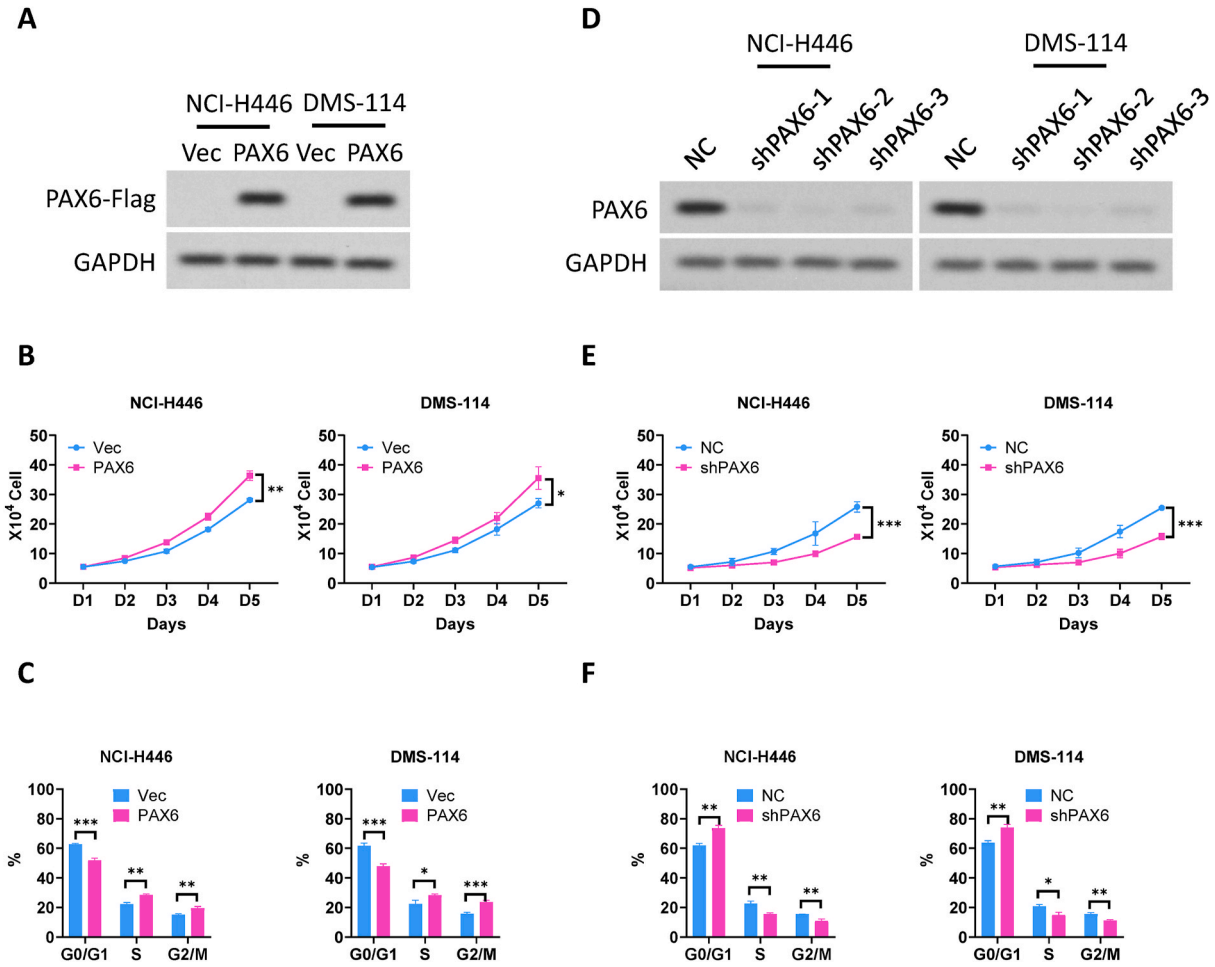

Fig. 1. PAX6 promotes proliferation and cell cycle progression in SCLC. (A) to (C) PAX6-Flag or control vector was stably expressed in NCI-H446 and DMS-114 cells. Western blot analysis (A), cell counting assay (B), and flow cytometry analysis of cell cycle (C) were performed. (D) to (F) Three different shRNA against PAX6 or control shRNA were stably expressed in NCI-H446 and DMS-114 cells. Western blot analysis (D), cell counting assay (E), and flow cytometry analysis of cell cycle (F) were performed. The bar represents mean ± SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

2.8. Apoptosis analysis

Cell apoptosis was detected by Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). The cells were suspended again in a binding buffer, with a volume of 500 μl per sample. The cells were subsequently labeled with Annexin-V-FITC (5 μl per sample).

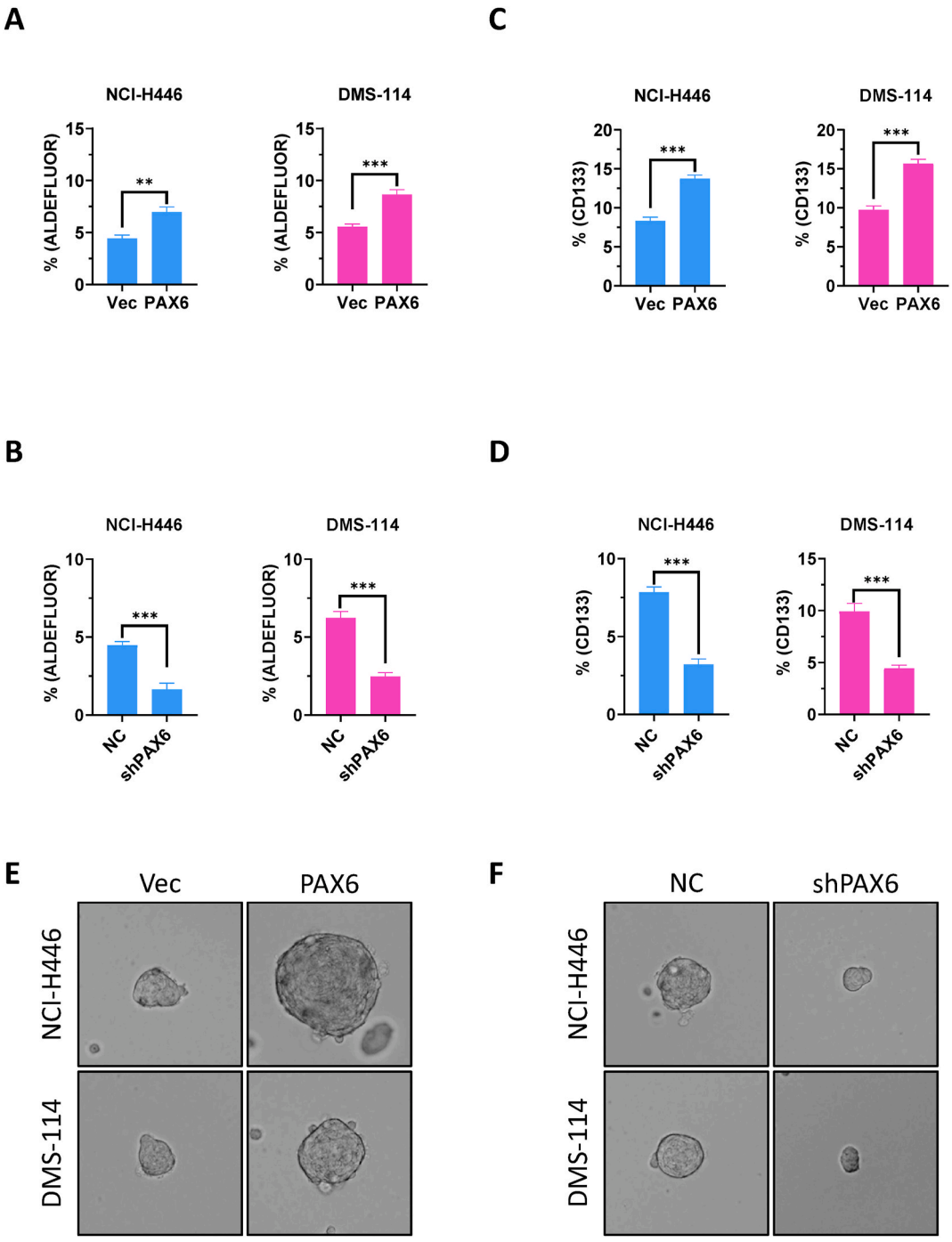

Fig. 2. PAX6 is critical for cancer stemness properties in SCLC. (A), (C) and (E) PAX6-Flag or control vector was stably expressed in NCI-H446 and DMS-114 cells. Cells were subjected to flow cytometry analysis of ALDEFLUOR (A) and CD133 (C) and sphere formation assay (E). (B), (D) and (F) ShRNA against PAX6 or control shRNA were stably expressed in NCI-H446 and DMS-114 cells. Cells were subjected to flow cytometry analysis of ALDEFLUOR (B) and CD133 (D) and sphere formation assay (F). The bar represents mean ± SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

Ultimately, the cells were dyed with PI (1μl/sample). Following the aforementioned incubation period, the cells underwent flow cytometry analysis.

2.9. CD133 analysis

The cells were collected and suspended in cold PBS at a density of 1×10^6 cells per 100 μl. The cells (100 μl) were exposed to 5 μl of CD33-FITC antibody and kept on ice for 30 min. Subsequently, the cells underwent flow cytometric analysis.

2.10. Cell counting assay

The cells were subjected to drug treatment at the specified dose and duration, and thereafter underwent trypsinization. A volume of 50 μL of cells was combined with an equal volume of 0.4 % trypan blue using a gentle pipetting technique. Each chamber of the hemocytometer was filled with 20 μL of the mix. The counting was conducted in duplicate by a single analyzer using a 40 × objective.

2.11. Cell cycle analysis

A suspension of single cells was prepared. The cells were fixed in 70 % ethanol at a temperature of −20 °C for the duration of one night. The cells were rinsed twice with phosphate-buffered saline (PBS). The cells were stained using a 1 ml solution of propidium iodide (PI, 50 μg/ml, Sigma) and RNase A (0.5 μg/ml, Sigma) for a duration of 30 min. Flow cytometry was used to evaluate the cells.

2.12. Statistical analysis

The statistical analysis was conducted with SPSS 16.0. The Student's t-test was employed to compare two groups. The significance level was established at a P-value of less than 0.05. The bar graphs and line graphs were conducted with GraphPad Prism (GraphPad software). All data shown represent the results obtained from triplicated independent experiments with standard deviation of the mean (mean ± SD). Degrees of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) are depicted in the respective figure legends.

3. Results

3.1. PAX6 promotes proliferation and cell cycle progression in SCLC

In order to examine the role of PAX6 in SCLC, our initial focus was on assessing the impact of PAX6 on the proliferation of SCLC cells. The expression of PAX6 was increased in the SCLC cell lines NCI-H446 and DMS-114, as shown in Fig. 1A. Our analysis demonstrated that the increased expression of PAX6 resulted in enhanced proliferation of SCLC cells, as compared to the control group treated with a vector (Fig. 1B). Flow cytometry analysis revealed that the overexpression of PAX6 in SCLC cells led to a reduction in the G1 phase population and an increase in the S and G2/M phase populations (Fig. 1C and Supplementary Fig. 1A). We suppressed the expression of PAX6 to assess its impact on cell growth. As shown in Fig. 1D, lentivirus-mediated gene knockdown resulted in the downregulation of PAX6 expression in SCLC cells, and shPAX6-2 was applied for the following experiments. Our data demonstrated that the use of PAX6-specific shRNA suppressed the proliferation of SCLC cells in comparison to the control shRNA that does not target any specific gene (Fig. 1E). Consistently, the decrease in PAX6 led to an increase in G1 phase arrest and a decrease in the population of cells in the S and G2/M phases (Fig. 1F and Supplementary Fig. 1B). The research provided evidence that PAX6 acted as an oncogenic regulator, controlling the growth of cells in SCLC. We also examined the role of PAX6 on cell proliferation and cell cycle in immortalized human bronchial epithelial (HBE) cells. Our data confirmed that upregulation of PAX6 enhanced cell proliferation and promoted cell cycle progression in HBE cells (Supplementary Figs. 1C–1E), while downregulation of PAX6 decreased cell proliferation and inhibited cell cycle progression in HBE cells (Supplementary Figs. 1G–1I).

3.2. PAX6 is critical for cancer stemness properties in SCLC

Cancer stem cells play a crucial role in the emergence of treatment resistance [6]. We assessed the potential involvement of PAX6 in the modulation of CSC characteristics in SCLC. Fig. 2A and Supplementary Fig. 2A demonstrated that the increase in PAX6 expression resulted in an elevation of the ALDEFLUOR+ population in NCI-H446 and DMS-114 cells. In contrast, the suppression of PAX6 expression resulted in a decrease in the ALDEFLUOR+ population in NCI-H446 and DMS-114 cells (Fig. 2B and Supplementary Fig. 2B). CD133 expression is another marker of CSC in SCLC. The impact of PAX6 on CSCs was verified through the assessment of the CD133+ population. Our observations indicated that the rise in PAX6 expression led to an increase in the CD133+ population in NCI-H446 and DMS-114 cells (Fig. 2C and Supplementary Fig. 2C). Conversely, the suppression of PAX6 expression resulted in a decrease in the CD133+ population in NCI-H446 and DMS-114 cells (Fig. 2D and Supplementary Fig. 2D). In HBE cells, we also found that PAX6 was consistently important for the regulation of CD133+ population (Supplementary Figs. 1F and 1J). In addition, sphere-forming assays were employed to assess the in vitro features of CSCs. The overexpression of PAX6 greatly increased the capacity of NCI-H446 and DMS-114 cells to form spheres (Fig. 2E), whereas the suppression of PAX6 expression dramatically reduced the ability of NCI-H446 and DMS-114 cells to produce spheres (Fig. 2F). Therefore, our findings indicated that PAX6 may play a crucial role in regulating the characteristics of CSCs in SCLC.

3.3. Inhibition of drug-induced apoptosis by PAX6 leads to enhanced drug resistance in SCLC

We subsequently investigated whether PAX6 exerted control over apoptosis in SCLC cells. Manipulation of PAX6 expression levels, whether increased or decreased, did not trigger cell death or activate the cellular pathway leading to programmed cell death in NCI-H446 cells (Fig. 3A–D and Supplementary Figs. 3A and B). Furthermore, similar phenomena were also observed in DMS-114 cells (Fig. 3E, F and Supplementary Figs. 3C and D). We conducted additional analysis to determine if PAX6 influenced drug sensitivity. Fig. 3A, E and Supplementary Fig. 3A, C demonstrated that cisplatin treatment effectively triggered apoptosis in NCI-H446 and DMS-114 cells. However, the overexpression of PAX6 significantly reduced the extent of cisplatin-induced apoptosis in both NCI-H446 and DMS-114 cells. Consistently, elevated expression of PAX6 also counteracted the activation of the apoptotic pathway generated by cisplatin in NCI-H446 and DMS-114 cells (Fig. 3B–D). Furthermore, the decrease in PAX6 expression greatly intensified cisplatin-induced cell death and the activation of the apoptotic pathway (Fig. 3C, D and Supplementary Fig. 3B). The data provided evidence that PAX6 played a crucial role in controlling drug sensitivity in SCLC. We additionally verified that the expression of PAX6 had an impact on the drug sensitivity of NCI-H446 cells to etoposide, as demonstrated in Fig. 3G, H and Supplementary Figs. 3E and F.

3.4. Nanog is critical for PAX6 regulated biological function in SCLC

We next determined how PAX6 regulated oncogenic function in SCLC. OCT4, SOX2, KLF4, c-Myc, and Nanog were demonstrated to play a critical role in maintaining stemness [19,20]. We examined whether PAX6 might regulate the expression of OCT4, SOX2, KLF4, c-Myc, and Nanog. As shown in Fig. 4A, upregulation of PAX6 expression promoted Nanog expression, but not OCT4, SOX2, KLF4, and c-Myc expression in NCI-H446 and DMS-114 cells. On the contrary, the downregulation of PAX6 expression suppressed Nanog expression, but not OCT4, SOX2, KLF4, and c-Myc expression in NCI-H446 and DMS-114 cells (Fig. 4B). We further explored whether Nanog played a critical role in mediating PAX6-induced oncogenic function in SCLC. Nanog shRNA or control shRNA was transfected in SCLC cell expressed PAX6 or vector (Fig. 4C). Our observations indicated that the decrease in Nanog expression eliminated the ability of PAX6 to decrease the number of cells in the G1 phase and increase the number of cells in the S and G2/M phases in NCI-H446 and DMS-114 cells (Fig. 4D and Supplementary Fig. 4A). Consistently, the reduction of Nanog prevented the increase of the ALDEFLUOR+ population produced by PAX6 in NCI-H446 and DMS-114 cells (Fig. 4E and Supplementary Fig. 4B). In addition, the expression of Nanog eradicated the chemoresistance produced by PAX6 against cisplatin in NCI-H446 and DMS-114 cells (Fig. 4F and Supplementary Fig. 4C). The data indicated that Nanog played a crucial role in the oncogenic function regulated by PAX6 in SCLC.

3.5. PAX6 suppresses NOTCH signaling to promote Nanog expression in SCLC

It has been demonstrated that NOTCH signaling activation greatly inhibits the proliferation of SCLC cells by causing a G2-M cell cycle arrest and the accumulation of sub-G1 populations [21,22]. We conducted an experiment to investigate the potential role of PAX6 in the regulation of NOTCH signaling activity. Fig. 5A demonstrated that the increased expression of PAX6 inhibited the expression of NOTCH intracellular domain (NICD) and HEY-1 in NCI-H446 and DMS-114 cells. This suggested that the activation of NOTCH signaling is regulated by PAX6. We maintained the continued activation of NOTCH signaling by expressing exogenous NICD (Fig. 5A). Interestingly, the expression of exogenous NICD eliminated the expression of Nanog caused by PAX6 (Fig. 5A). We conducted additional analysis to determine if the activation of NOTCH signaling was essential for the oncogenic activities produced by PAX6. Fig. 5B demonstrated that the activation of NOTCH signaling in NCI-H446 cells impaired the ability of PAX6 to stimulate cell growth. In the same way, the activation of NOTCH signaling eliminated the cell cycle progression bolstered by PAX6 (Fig. 5C and Supplementary Fig. 5A). In addition, the overexpression of exogenous NICD also negated the enrichment of the ALDEFLUOR+ population caused by PAX6 (Fig. 5D and Supplementary Fig. 5B), and mitigated the chemoresistance induced by PAX6 in NCI-H446 cells (Fig. 5E and Supplementary Fig. 5C). The data demonstrated that the activity of NOTCH signaling was crucial for the oncogenic function mediated by PAX6 in SCLC.

4. Discussion

The strong invasiveness, high degree of malignancy, and propensity for therapeutic resistance of SCLC have resulted in several fatalities [23]. At first, the majority of patients with SCLC have a positive response to first treatment with chemotherapy that includes platinum-based drugs. Nevertheless, patient reactions frequently lack long-term efficacy, and the emergence of chemotherapy resistance is frequently observed, leading to the recurrence of the disease [23]. The absence of specific therapeutic options for individuals afflicted with SCLC compels the exploration of novel, logical targeted therapies. In the present study, we explored the role of PAX6 in SCLC. Our data showed that PAX6 functioned as an oncogene to promote proliferation, cell cycle progression, drug resistance, and CSC properties. Given that CSCs play a key role in tumor initiation, development, and therapeutic resistance, a deeper comprehension of the molecular pathways essential to CSC expansion and maintenance may help to enhance clinical management of SCLC [5]. Here, we show that targeting NOTCH pathway might be an potential strategy to suppress the PAX6-NOTCH-Nanog signaling axis to eradicate CSCs and overcome drug resistance. The NOTCH pathway mediates decisions about cell fate and, depending on the situation, can be either tumor suppressive or oncogenic [24]. The NOTCH pathway is critical for the regulation of the malignant behavior of SCLC [25]. Targeting NOTCH pathway, such as NOTCH ligand DLL3, acts as a promising strategy for precision therapy in SCLC [25]. Our data consistently shows that targeting NOTCH pathway might reduce CSCs to overcome drug resistance. Importantly, we present a novel mechanism that targets NOTCH to impair PAX6-induced Nanog expression.

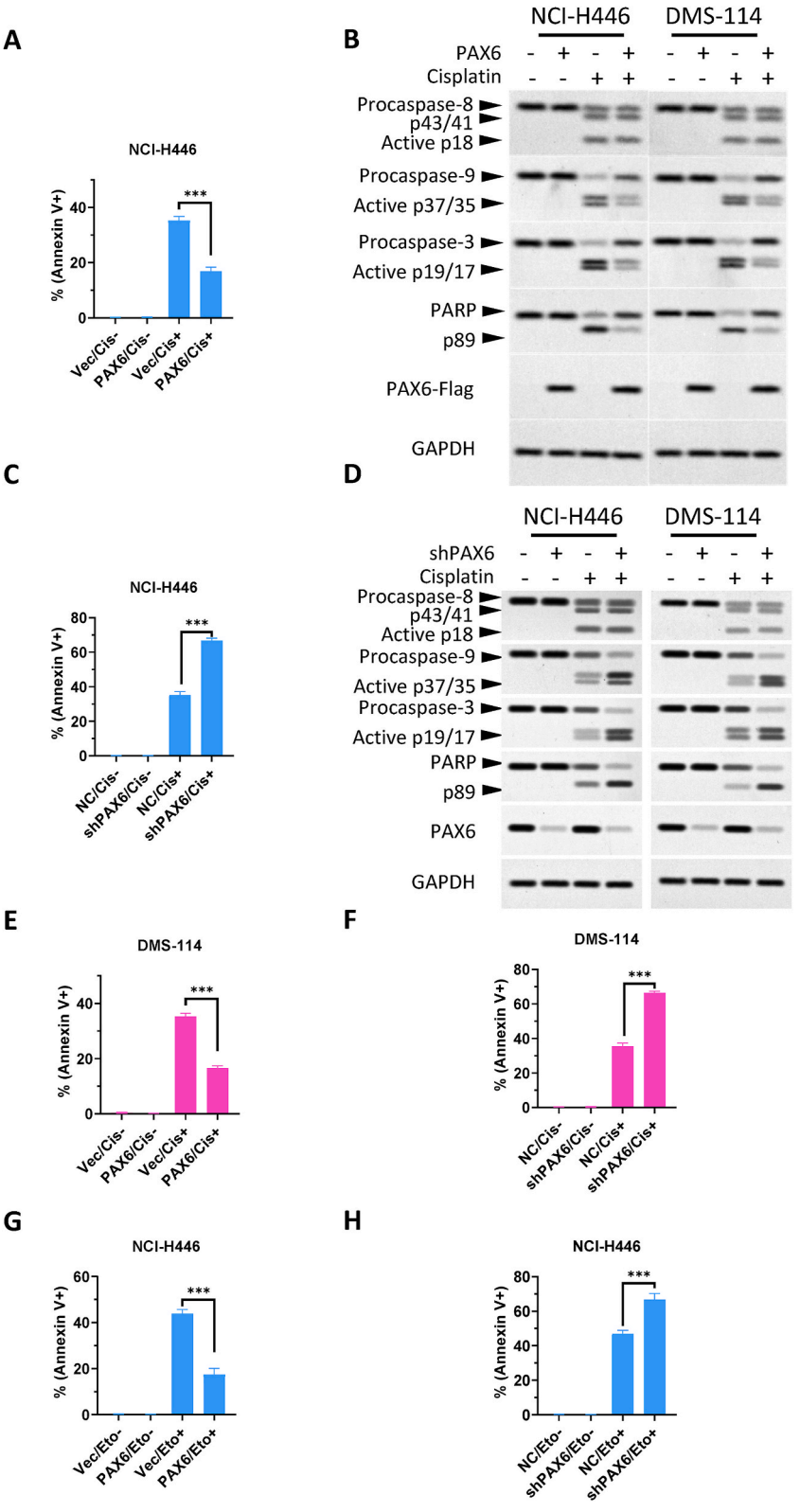

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Fig. 3. Inhibition of drug-induced apoptosis by PAX6 leads to enhanced drug resistance in SCLC. (A) and (B) PAX6-Flag or control vector were stably expressed in NCI-H446 cells. Cells were treated with or without Cisplatin (0.5 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis (A) and Western blot analysis (B). (C) and (D) ShRNA against PAX6 or control shRNA were stably expressed in NCI-H446. Cells were treated with or without Cisplatin (0.5 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis (C) and Western blot analysis (D). (E) PAX6-Flag or control vector were stably expressed in DMS-114 cells. Cells were treated with or without Cisplatin (0.5 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis. (F) ShRNA against PAX6 or control shRNA were stably expressed in DMS-114 cells. Cells were treated with or without Cisplatin (0.5 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis. (G) PAX6-Flag or control vector were stably expressed in NCI-H446 cells. Cells were treated with or without Etoposide (1 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis. (H) ShRNA against PAX6 or control shRNA were stably expressed in NCI-H446. Cells were treated with or without Etoposide (1 μM) for 48h. Cells were harvested for flow cytometry analysis of apoptosis. The bar represents mean ± SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

PAX6 plays an important role in the development process [7]. Recent studies suggestes that PAX6 is essential for the development of cancers. The expression and biological function of PAX6 in human cancer vary among various kinds of cancer. In glioblastoma [15] and prostate cancer [26], PAX6 is downregulated in tumors relative to normal tissues and functions as a tumor suppressor gene. In pancreatic cancer [10], on the other hand, it is overexpressed and works as an oncogene, promoting cell proliferation and suppressing terminal differentiation. These research indicated that the functional role of PAX6 is cellular context dependent in cancers. In lung cancer, it was shown that PAX6 enhances the growth and advancement of the cell cycle in human NSCLC cells [27]. Besides, PAX6-ZEB2 pathway enhances the metastasis and drug resistance to cisplatin in NSCLC via activating the PI3K/AKT signaling pathway [17]. Moreover, PAX6 is controlled by promoter methylation to promote cancer development via GLI-SOX2 signaling axis in NSCLC [11]. Furthermore, the expression of PAX6 has a crucial role in the promotion of cell migration, invasion, proliferation, and viability in NSCLC cells induced by SMAD3 [28]. However, the significance of PAX6 in SCLC remains mostly unknown. In this study, we provided the evidence which PAX6 accelerates CSC proliferation in SCLC and is a key oncogene responsible for cancer stemness properties. In vivo experiments are needed for further validating the oncogenic function of PAX6 in SCLC.

Pax6 participates in the regulation of stem cell function by modulating multiple aspects of neural stem cell and neuron development in the embryonic central nervous system [29]. In cancers, Pax6 might also have role in the regulation of CSC properties. The expression of PAX6 in NSCLC is regulated by the addition of methyl groups to its promoter region, which in turn increased the characteristics of CSCs via the GLI-SOX2 signaling pathway [11]. Furthermore, EMT phenotypic cells acquire stem-like cell signatures [30], PAX6 directly bound to the promoter region of ZEB2 to promote EMT-mediated stem cell transformation [17]. Additionally, Pax6 is over-expressed in sphere-forming cells and might regulate CSC properties in retinoblastoma [31]. In this study, we reported for the first time that PAX6 was critical for CSC properties regulation in SCLC. We showed that NOTCH-Nanog axis was required for PAX6 regulated CSC stemness in SCLC. NANOG, a crucial controller of embryonic development and cellular reprogramming, has been found to be widely present in human malignancies, critical for regulating CSC properties [32]. Though PAX6 is a important transcription factor in humans, it is yet unknown how exactly PAX6 controls the characteristics of CSCs in SCLC at the molecular level. Previous research has shown that PAX6 has the ability to bind to the promoter region of pluripotent genes [33], such as Oct4 and Nanog. This binding action leads to the suppression of pluripotent gene expression and ultimately causes the cells to transition out of the embryonic stem cell state [33]. However, other study show that PAX6 expression significantly positively correlates with the expression of pluripotent genes [17], such as NANOG, SOX2, and OCT4. In our study, we do not acquire the evidence that PAX6 directly binds and activates Nanog promoter, but we find that NOTCH pathway might mediate PAX6 regulated Nanog expression. Previous study shows that NOTCH pathway activation is required for human embryonic stem cells (hESCs) to initiate embryonic germ layers differentiation [34]. Blocking NOTCH activation preserves undifferentiated hESCs and strongly enhances the expression of Nanog [34], indicating that NOTCH pathway might suppress Nanog expression during hESCs differentiation. Our data consistently validated the negative regulation effects of NOTCH on Nanog in SCLC. The susceptibility of NSCLC cells to cisplatin is reduced by the downregulation of pax6, which controls the transformation of stem cells in NSCLC [17]. The results of our investigation confirmed a positive correlation between PAX6 and NANOG. Additionally, our findings demonstrated that the PAX6-Nanog axis plays a crucial role in promoting CSC properties in SCLC. It will take further study to show whether PAX6's transcription function can directly control the expression of Nanog. Furthermore, targeting PAX6-NOTCH-Nanog axis in vivo experiments to observe the anti-cancer effects on SCLC is helpful for understanding the roles of PAX6-NOTCH-Nanog axis in SCLC.

In summary, our work showed that PAX6 plays a crucial role in regulating the NOTCH-Nanog signaling axis, which controls the characteristics of cancer stem cells in SCLC. Our findings may provide an approach for eliminating CSCs and surmounting treatment resistance in SCLC.

CRediT authorship contribution statement

Xiao-Fang Guo: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation. **Ling-Ling Wang:** Writing – original draft, Validation, Methodology, Formal analysis. **Fei-Meng Zheng:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **He-Ping Li:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Informed consent statement

Not applicable.

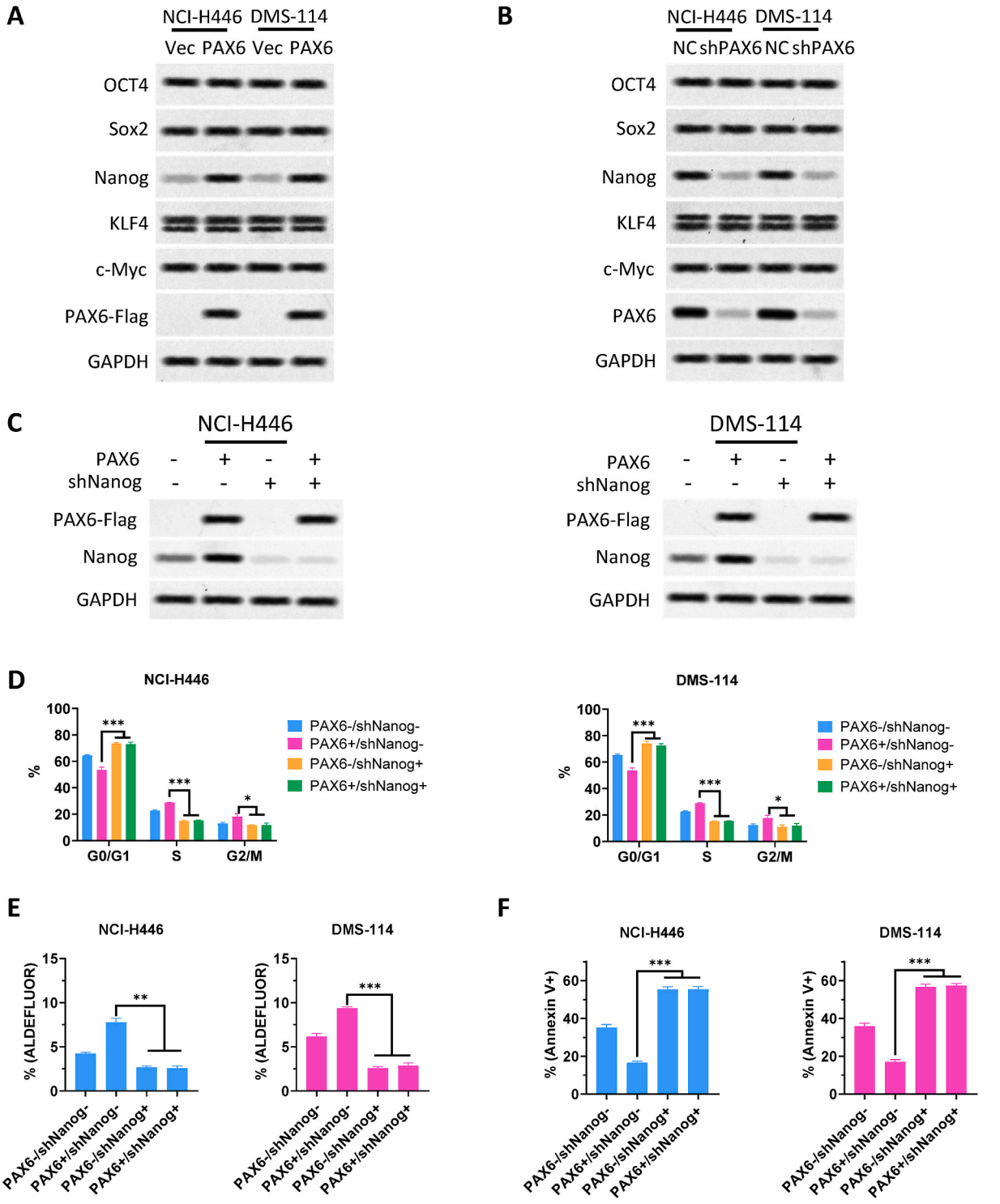

Fig. 4. Nanog is critical for PAX6 regulated biological function in SCLC. (A) PAX6-Flag or control vector was stably expressed in NCI-H446 and DMS-114 cells. The interested proteins were detected by Western blot analysis. (B) ShRNA against PAX6 or control shRNA were stably expressed in NCI-H446 and DMS-114 cells. The interested proteins were detected by Western blot analysis. (C) to (F) ShRNA against Nanog or control shRNA were expressed in PAX6 overexpressed or control NCI-H446 and DMS-114 cells. The interested proteins were detected by Western blot analysis (C). Cell cycle analysis (D), ALDEFLUOR analysis (E), and apoptosis analysis (F) were performed. The bar represents mean ± SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

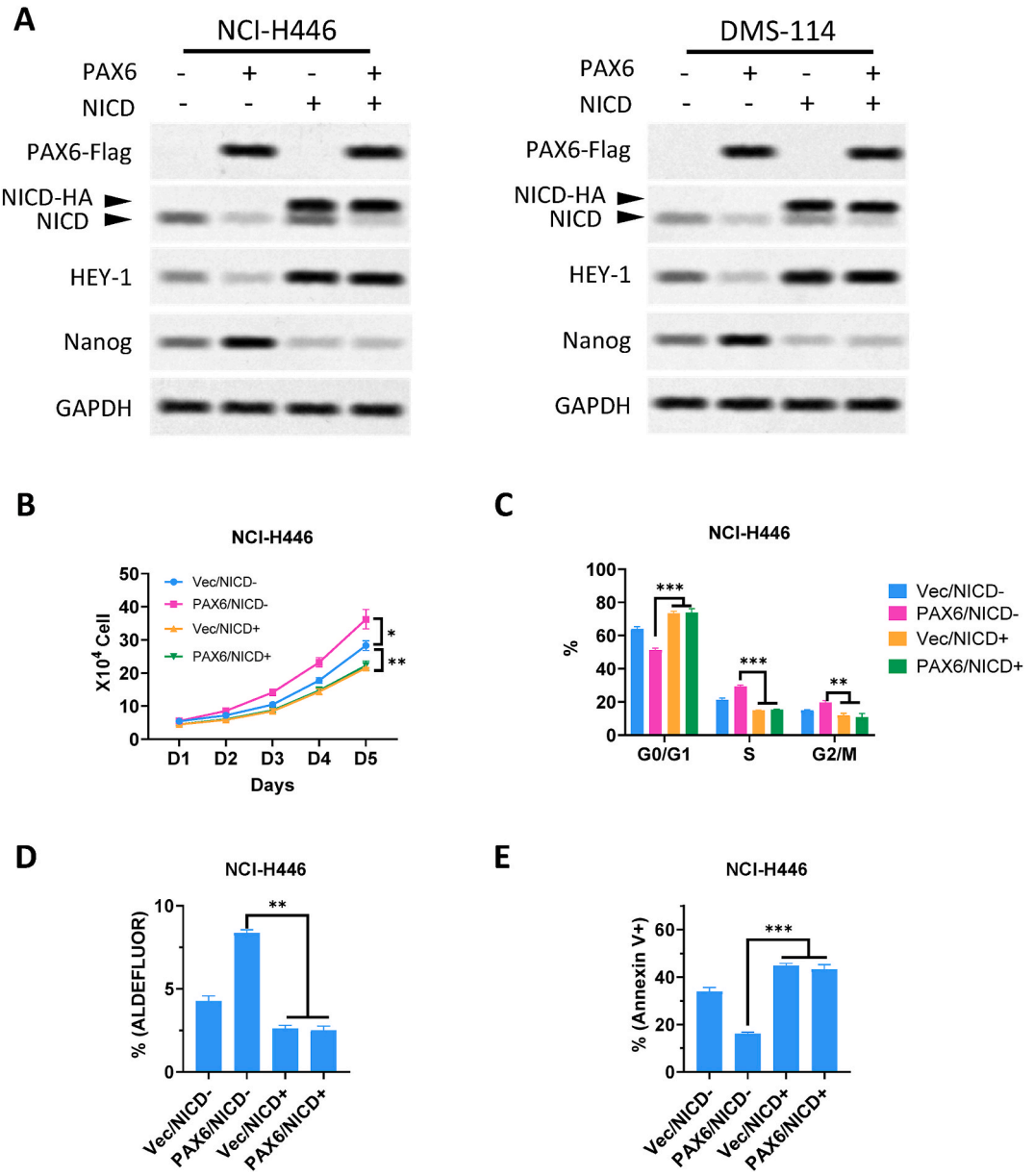

Fig. 5. PAX6 suppresses NOTCH signaling to promote Nanog expression in SCLC. (A) to (E) NICD-HA was expressed in PAX6-Flag overexpressed or control NCI-H446 or DMS-114 cells. The interested proteins were detected by Western blot analysis (A) in NCI-H446 or DMS-114 cells. Cell counting assay (B), Cell cycle analysis (C), ALDEFLUOR analysis (D), and apoptosis analysis (E) were performed. The bar represents mean ± SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

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Declaration of competing interest

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Abbreviations

SCLC	Small cell lung cancer
CSC	Cancer stem cell
NSCLC	Non-small cell lung cancer
PAX6	Paired-box 6
ATCC	American Type Culture Collection
HRP	Horseradish peroxidase
DEAB	N,N-diethylaminobenzaldehyde
PBS	Phosphate-buffered saline
PI	Propidium iodide
LSD	Least significant difference
NICD	NOTCH intracellular domain
EMT	Epithelial-mesenchymal transition

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41795.

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