

## ORIGINAL RESEARCH

# VGLL4 inhibits stemness and cisplatin resistance in non-small cell lung cancer *via* the COL3A1/NF- $\kappa$ B pathway

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**Abstract**

Previous studies have confirmed that vestigial-like protein 4 (VGLL4) can inhibit the malignant progression of lung cancer cells. However, its impact on cisplatin resistance and stemness in lung cancer cells remains unclear. In this study, we established cisplatin-resistant cells and transfected them with VGLL4 overexpression plasmid and siRNA. Their 50% inhibitory concentration (IC<sub>50</sub>) values were determined *via* Cell Counting Kit-8 (CCK-8) assay, cell proliferation was assessed *via* clone formation assay, apoptosis rate was measured by flow cytometry, sphere formation was quantified, and protein expression of collagen type III alpha 1 (COL3A1) and p-p65/p65 was analyzed using Western blot. Our findings demonstrate that VGLL4 enhances the sensitivity of cisplatin-resistant cells to cisplatin, inhibits cell proliferation, and promotes apoptosis. Moreover, VGLL4 suppresses sphere formation and the expression of stemness markers Nanog and Oct4 in cisplatin-resistant cells. Mechanistically, VGLL4 regulates the nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) pathway through COL3A1, thereby influencing the sensitivity and stemness characteristics of cisplatin-resistant cells. In conclusion, this study shows that VGLL4 can augment treatment sensitivity and suppress stemness of cisplatin-resistant cells, thereby proposing a potential therapeutic target for cisplatin-resistant lung cancer.

**Keywords**

Lung cancer; VGLL4; Cisplatin resistance; Stemness; COL3A1; NF- $\kappa$ B

## 1. Introduction

Lung cancer is one of the most common malignant tumors worldwide and is associated with high cancer-related mortality. Non-small cell lung cancer (NSCLC) accounts for a significant portion of lung cancer diagnoses, comprising approximately 80% of all LC cases. The primary mechanism of action of many clinically used anticancer drugs involves disrupting DNA replication or inducing DNA damage to trigger cell apoptosis, including the widely employed cisplatin (DDP). However, the efficacy of cisplatin treatment is often impeded by drug resistance [1, 2], indicating that further understanding on the underlying causes of cisplatin resistance is essential for improving treatment outcomes. In this regard, it has been proposed that cancer stem cells (CSCs) play a pivotal role in tumor progression, metastasis, and resistance to therapy [3, 4]. Thus, identifying potential targets associated with cisplatin resistance and stemness in NSCLC is urgently needed to advance cisplatin resistance treatment strategies.

Vestigial-like protein 4 (VGLL4) is a member of the vestigial-like family and functions as a tumor suppressor. It has been demonstrated that VGLL4 depletion inhibits the proliferation, migration, invasion, and epithelial-mesenchymal

transition of NSCLC cells [5], and its upregulation suppresses the proliferation, stemness, invasion, and migration of epidermal squamous cell carcinoma. Additionally, VGLL4 overexpression has been correlated with reduced mRNA and protein levels of COL3A1 [6].

COL3A1, a fibrillar collagen, is overexpressed in various tumors. Previous research has identified elevated expression of COL3A1 in cisplatin-resistant lung cancer cells, with knock-down of COL3A1 correlating with increased sensitivity to cisplatin [7]. Furthermore, COL3A1 has been related to poor prognosis and malignant progression by activating the NF- $\kappa$ B pathway in tumor cell carcinoma [8].

NF- $\kappa$ B is a pathway associated with tumor cisplatin resistance and development, and inhibition of NF- $\kappa$ B has been shown to augment the anti-tumor efficacy of cisplatin in resistant cell lines. Moreover, NF- $\kappa$ B signaling is closely associated with the stemness phenotype of CSCs, and activation of NF- $\kappa$ B signaling, characterized by increased nuclear translocation of NF- $\kappa$ B p65, has been shown to promote stemness features such as enhanced sphere formation, migration, chemoresistance and expression of stemness markers [9, 10].

In this study, we present promising insights into the role of VGLL4 in NSCLC cisplatin resistance and stemness by

elucidating that VGLL4 exerts inhibitory effects on cisplatin resistance and tumor stemness by downregulating COL3A1, thereby suppressing the NF- $\kappa$ B pathway. Thus, proposing VGLL4 as a modulator of NSCLC cisplatin resistance suggests a promising avenue for therapeutic intervention and enhances our understanding of the underlying mechanisms driving this clinical challenge.

## 2. Method

### 2.1 Cell culture

NSCLC cell lines, A549 and H1299, were purchased from the Typical Cultures Depository Center (USA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixture. Following previously established protocols, A549 and H1299 cells were exposed to incrementally rising concentrations of cisplatin to induce resistance. The resultant cisplatin-resistant cells were designated as A549/DDP and H1299/DDP, respectively. The cisplatin-resistant characteristics were maintained by culturing the cells with 1  $\mu$ g/mL DDP supplemented in the medium.

### 2.2 Cell transfection

siRNA expression vectors and corresponding negative control vectors targeting VGLL4 were synthesized by Genepharma (Shanghai, China). The sequences were as follows: siRNA-negative control (si-NC) (5'-CUAGAACUGGACAACGACA-3'); si-VGLL4 (5'-AGGACCTAGACTGTGACAA-3'). For overexpression of VGLL4, the VGLL4 cDNA was cloned into an appropriate vector. Transfection of siRNA or overexpression plasmids into cells was achieved using Lipofectamine 3000 (L3000075, ThermoFisher, Waltham, MA, USA).

### 2.3 CCK8 assay

The IC<sub>50</sub> values of cisplatin on parental and resistant cells were assessed using CCK8 assays. Briefly, the cells were seeded into 96-well plates and exposed to varying concentrations of DDP. Then, the CCK8 reagent was added and incubated, following which cell viability was measured using an enzymatic marker at 450 nm to calculate their corresponding IC<sub>50</sub> values.

### 2.4 Clone formation assay

Cells in the logarithmic growth phase were trypsinized and resuspended in a cell suspension. Next, they were seeded into 6-well culture plates and cultured for 14 days until single clonal colonies containing more than 50 cells were visible. Following this, the cells in each well were fixed with 4% paraformaldehyde and washed with PBS. Then, the colonies were stained with crystal violet staining solution and counted using a microscope.

### 2.5 Cell apoptosis analysis

Lung cancer cells were trypsinized, resuspended in 1  $\times$  binding buffer, and incubated with Annexin-V-FITC and propidium iodide (PI) in the dark to assess apoptosis. Flow cytometry was employed for the detection of apoptotic cells [11].

## 2.6 Sphere formation assays

The cells were seeded into 6-well plates and cultured in serum-free Dulbeccos Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) medium supplemented with B27, Epidermal Growth Factor (EGF) and basic fibroblast growth factor (bFGF). After 7–14 days of incubation, the resulting spheroids were counted using a microscope [12].

## 2.7 Western-blot

Cellular proteins were initially extracted by incubating the cells on ice with Radio-Immunoprecipitation Assay (RIPA) lysate. After adequate lysis, the cell lysates were centrifuged for 3–5 minutes, and the resulting supernatant was collected [13, 14]. Then, a Bovine Serum Albumin (BSA) standard system was prepared, and the protein concentrations of the samples were determined based on the standard curve and sample dilution. The proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk powder to minimize non-specific binding, the PVDF membranes were incubated overnight at 4 °C with primary antibodies. Subsequently, the membranes were probed with secondary antibodies at room temperature. Lastly, protein bands were visualized using an enhanced chemiluminescence (ECL) luminescent solution. The primary antibodies used were as follows: VGLL4 (1:2500, ThermoFisher, China, Shanghai, 16H37L22), Nanog (1:1000, Affinity, China, Changzhou, Jiangsu, AF5388), Oct4 (1:1000, Affinity, AF0226), COL3A1 (1:1000, Affinity, AF5457), p-p65 (1:1000, Affinity, AF2006), p65 (1:1000, Affinity, AF5006), and  $\beta$ -actin (1:1000, Affinity, AF7018).

## 2.8 Statistical analysis

Statistical analyses were performed using Student's *t*-tests to evaluate differences between two groups. Comparisons among multiple groups were assessed by Analysis of Variance followed by *post hoc* pairwise comparisons. Data are presented as means  $\pm$  standard deviation (SD) from three independent experiments. A significance level of  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 VGLL4 enhances the sensitivity of lung cancer cells to cisplatin

Firstly, A549 and H1299 cisplatin-resistant cells were established by gradually increasing the concentration gradient. The IC<sub>50</sub> values were determined and calculated using the CCK8 assay, which showed significantly higher IC<sub>50</sub> values in the cisplatin-resistant cells compared to parental cells, thereby confirming the successful establishment of drug-resistant cells (Fig. 1A). Then, VGLL4 overexpression plasmid and siRNA were transfected into A549 and H1299 drug-resistant cells, respectively. The transfection efficiency was assessed *via* western blot analysis, which demonstrated a significant increase in VGLL4 protein expression in the VGLL4 overex-

pression group and a significant decrease in VGLL4 protein expression in the si-VGLL4 group, indicative of successful transfection (Fig. 1B). The impact of VGLL4 expression on cell proliferation and apoptosis was evaluated using clone formation assay and flow cytometry, respectively. The results indicated that VGLL4 overexpression significantly suppressed A549/DDP and H1299/DDP cell proliferation (Fig. 1C) and induced apoptosis (Fig. 1D). Conversely, si-VGLL4 exerted opposite effects to those observed in the VGLL4 overexpression group. Lastly, the influence of VGLL4 expression on drug-resistant cell sensitivity was assessed *via* CCK8 assay. The IC50 value of the VGLL4 overexpression group was found to be significantly higher than that of the vector group, suggesting enhanced sensitivity of A549/DDP and H1299/DDP cells to cisplatin upon VGLL4 upregulation, and opposite effects were observed using si-VGLL4 (Fig. 1E).

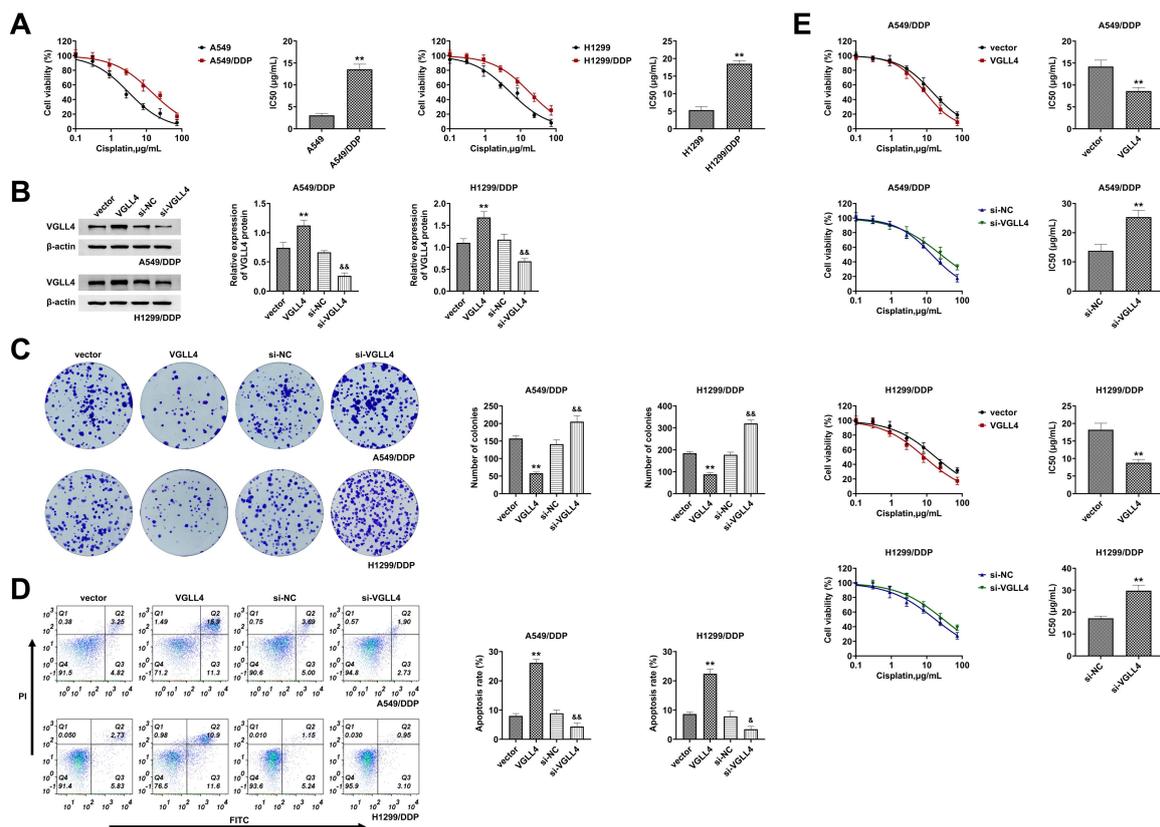
### 3.2 VGLL4 inhibits stemness characteristics of lung cancer cells

To investigate the impact of VGLL4 expression on stemness in drug-resistant cells, we conducted a sphere formation assay. The results demonstrated a significant reduction in the number

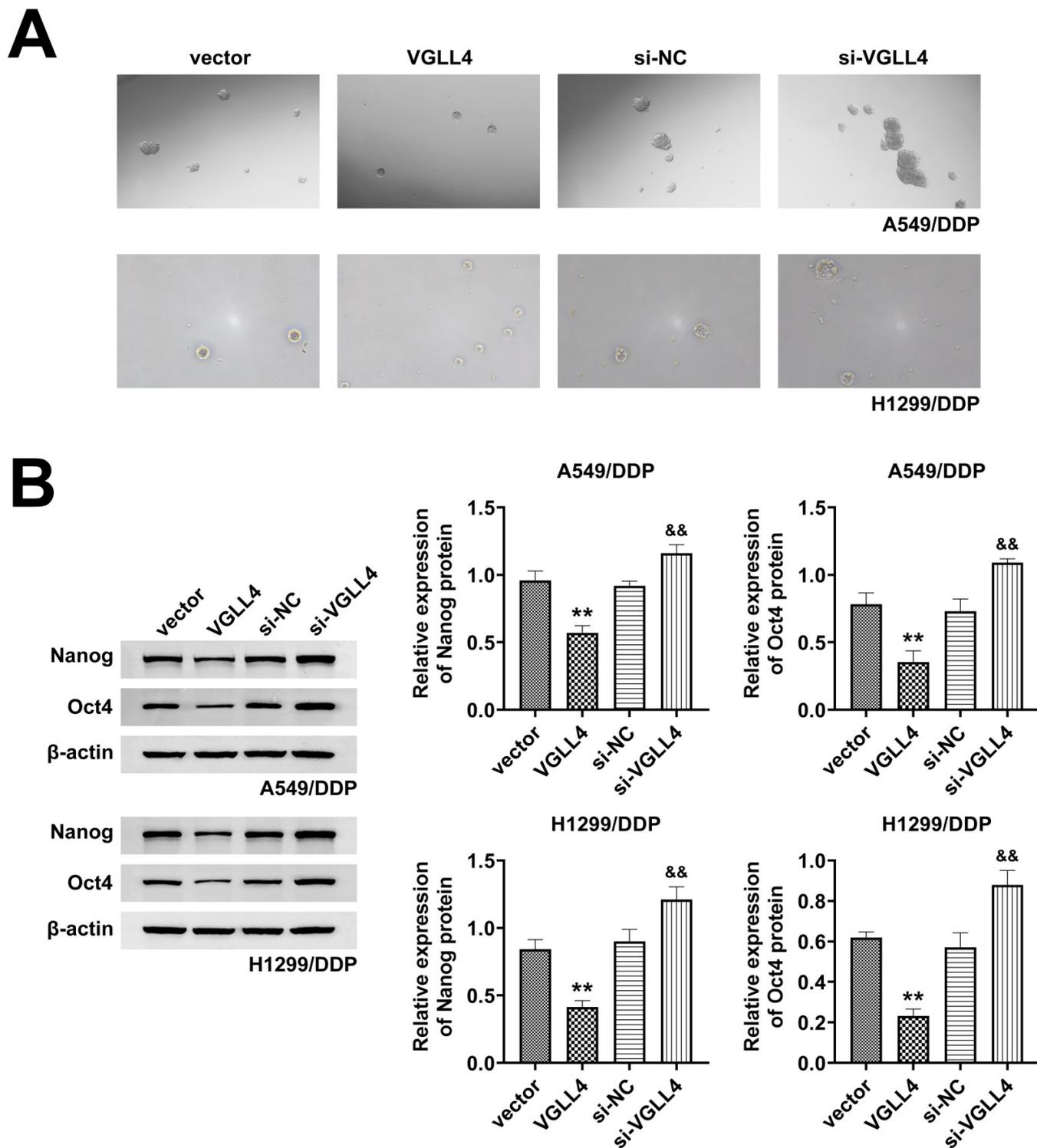
of sphere formations in the VGLL4 overexpression group compared to the control group (Fig. 2A). Additionally, the expression levels of stemness-associated markers Nanog and Oct4 proteins were found to be significantly decreased in the VGLL4 overexpression group (Fig. 2B), and si-VGLL4 yielded opposite effects. Overall, these findings suggest that VGLL4 could indeed suppress the stemness characteristics of drug-resistant cells in lung cancer.

### 3.3 VGLL4 regulates the NF- $\kappa$ B pathway through COL3A1

To determine the mechanistic role of VGLL4 in lung cancer drug resistance and stemness, western blot experiments and analyses were conducted to assess the expression of COL3A1 and p-p65/p65. Our results revealed that VGLL4 significantly reduced the protein expression of COL3A1 and p-p65/p65, whereas si-VGLL4 increased their expression (Fig. 3). Furthermore, co-transfection of si-VGLL4 and si-COL3A1 resulted in the reversal of si-VGLL4-mediated promotion of p-p65/p65 protein expression, suggesting that VGLL4 regulates the NF- $\kappa$ B pathway through COL3A1.



**FIGURE 1. VGLL4 enhances lung cancer cell sensitivity to cisplatin.** (A) IC50 values of lung cancer cells (A549, H1299) and their cisplatin-resistant counterparts (A549/DDP, H1299/DDP) were determined using the CCK8 assay. (B) Western blot analysis of VGLL4 protein expression in lung cancer cisplatin-resistant cells (A549/DDP, H1299/DDP) following transfection with VGLL4 or si-VGLL4. (C) Clone formation assay assessing proliferation of A549/DDP and H1299/DDP cells transfected with VGLL4 or si-VGLL4. (D) Flow cytometry analysis of apoptosis in A549/DDP and H1299/DDP cells transfected with VGLL4 or si-VGLL4. (E) Determination and calculation of IC50 values for A549/DDP and H1299/DDP cells using the CCK8 method. \*\* $p < 0.01$  compared with vector group; & $p < 0.05$ , && $p < 0.01$  compared with si-NC group. VGLL4: vestigial-like protein 4; si-NC: siRNA-negative control.

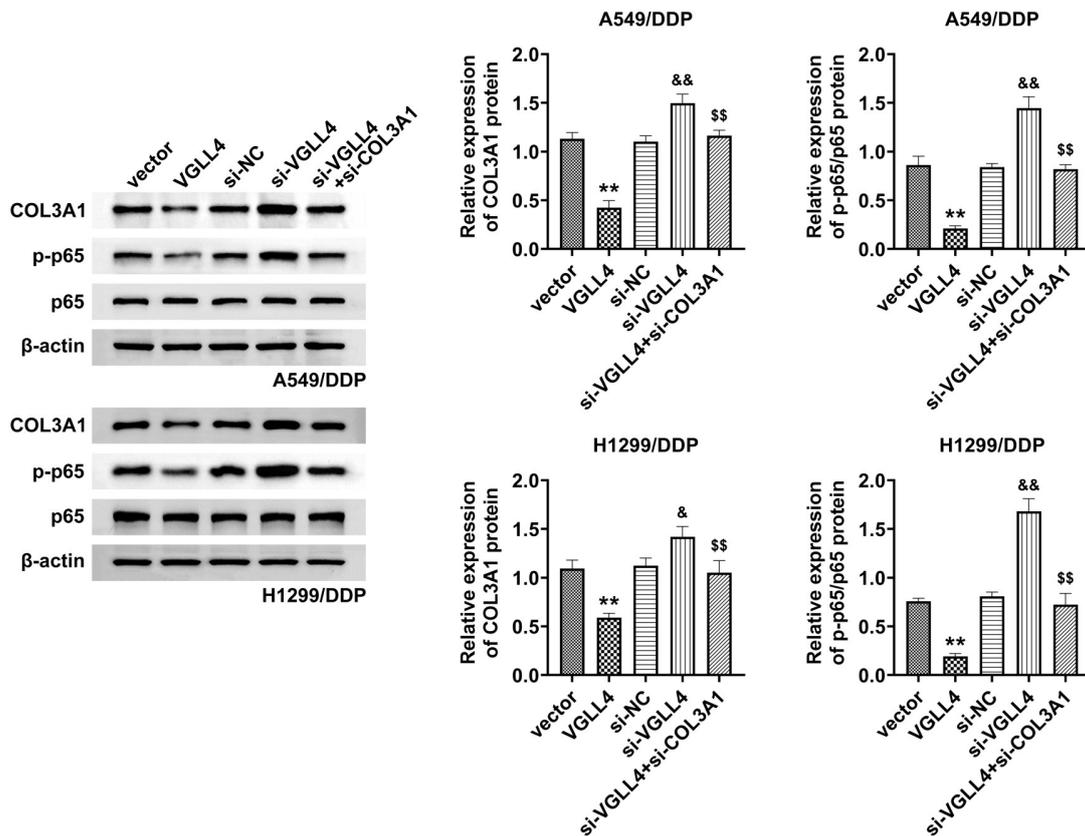


**FIGURE 2. VGLL4 inhibits the stemness characteristics of lung cancer cells.** (A) Sphere formation assay quantifying the number of spheres in A549/DDP and H1299/DDP cells transfected with VGLL4 or si-VGLL4. (B) Western blot analysis of protein expression levels of stemness markers Nanog and Oct4 in A549/DDP and H1299/DDP cells transfected with VGLL4 or si-VGLL4. \*\* $p < 0.01$  compared with vector group; && $p < 0.01$  compared with si-NC group. VGLL4: vestigial-like protein 4; DDP: cisplatin; si-NC: siRNA-negative control.

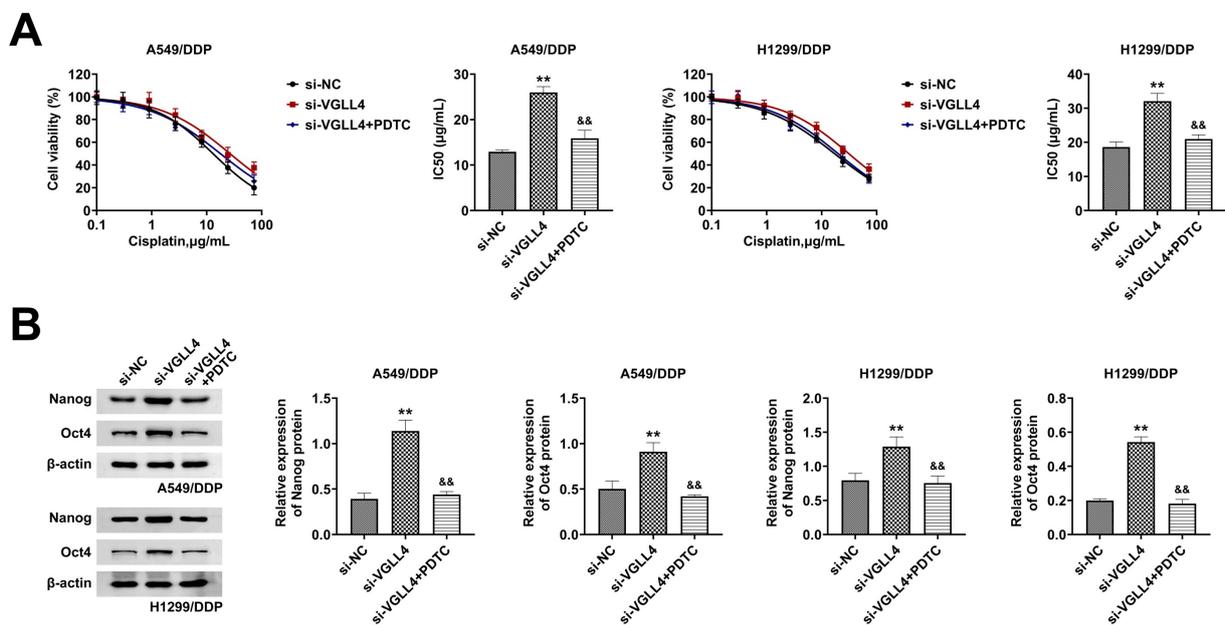
### 3.4 VGLL4 affects drug resistance and stemness of lung cancer cells through NF- $\kappa$ B

Following transfection with si-VGLL4, the cells were treated with the NF- $\kappa$ B inhibitor Pyrrolidinedithiocarbamate (PDTTC), and the results demonstrated that the si-VGLL4-induced in-

crease in IC<sub>50</sub> values (Fig. 4A) and the expression of stemness markers Nanog and Oct4 proteins (Fig. 4B) were reversed by PDTTC, indicating suggest that VGLL4 influences drug resistance and stemness of tumor cells through the NF- $\kappa$ B pathway.



**FIGURE 3. VGLL4 regulates the COL3A1/NF- $\kappa$ B pathway.** Western blot analysis of COL3A1 and p-p65 expression in A549/DDP and H1299/DDP cells transfected with VGLL4, si-VGLL4, and si-VGLL4 + COL3A1. \*\* $p < 0.01$  compared with vector group; & $p < 0.05$ , && $p < 0.01$  compared with si-NC group; \$\$ $p < 0.01$  compared with si-VGLL4 group. VGLL4: vestigial-like protein 4; DDP: cisplatin; COL3A1: si-NC: siRNA-negative control.



**FIGURE 4. VGLL4 affects drug resistance and stemness of tumor cells via the NF- $\kappa$ B pathway.** (A) CCK8 assay and determination of IC50 values for cisplatin-resistant cells transfected with si-VGLL4 or si-VGLL4 + PDTTC. (B) Western blot analysis of Nanog and Oct4 protein expression in cisplatin-resistant cells transfected with si-VGLL4 or si-VGLL4 + PDTTC. \*\* $p < 0.01$  compared with si-NC group; && $p < 0.01$  compared with si-VGLL4 group. VGLL4: vestigial-like protein 4; si-NC: siRNA-negative control; PDTTC: Pyrrolidinedithiocarbamate.

## 4. Discussion

VGLL4 downregulation has been observed across various cancer types, where it functions as a tumor suppressor gene, including prostate cancer [15], bladder cancer [16], gastric cancer [17], and colorectal cancer [18]. Previous investigations have indicated low expression levels of VGLL4 in both mouse and human lung cancer specimens, with documented inhibitory effects on lung cancer growth [19]. In this study, we investigated the role of VGLL4 in cisplatin-resistant cells and demonstrated its ability to enhance the sensitivity of lung cancer cisplatin-resistant cells to cisplatin. Furthermore, VGLL4 was found to suppress the tumor stem cell population within lung cancer drug-resistant cells. These findings collectively suggest that VGLL4 may represent a promising therapeutic target for addressing cisplatin resistance in lung cancer.

Consistent with our findings, previous research has indicated low expression levels of VGLL4 in drug-resistant breast cancer cells. Moreover, overexpression of VGLL4 has been demonstrated to exert a significant inhibitory effect on the proliferation of tamoxifen-resistant cells [20]. Additionally, in hepatocellular carcinoma cells, VGLL4 overexpression has been shown to counteract the effects of miR-301a-3p mimics, resulting in reduced proliferation, invasion, and chemotherapy resistance [21].

Extracellular matrix components, including collagen COL3A1, have been demonstrated to be expressed in tumor tissues and drug-resistant cell lines. Additionally, interactions between cancer cells and these ECM components have been shown to contribute to the development of drug resistance in cancer, which may change the sensitivity of cancer cells to apoptosis and increase their resistance to drugs. Notably, COL3A1 expression is elevated in drug-resistant cell lines, with aldehyde dehydrogenase 1A1-positive cells, a common biomarker of CSCs, primarily responsible for its production [22]. In our study, we found that VGLL4 modulates the NF- $\kappa$ B signaling pathway through COL3A1.

NF- $\kappa$ B heterodimers function as transcription factors, regulating downstream target genes. Under normal conditions, NF- $\kappa$ B dimers are bound by I $\kappa$ B in the cytoplasm. Upon stimulation, I $\kappa$ B is phosphorylated, leading to dissociation from NF- $\kappa$ B dimers, which translocate to the nucleus, thereby promoting downstream gene transcription [23]. These downstream target genes encompass various tumor phenotypes, including proliferation, migration, survival, angiogenesis, and cell death. Given NF- $\kappa$ B's role in regulating oncogenes and pro-survival genes, this pathway has been implicated in tumorigenesis, progression, and chemotherapy resistance [24]. Studies have also demonstrated NF- $\kappa$ B activity in both lung cancer and precancerous lung lesions, correlating with chemoresistance [25]. In this present study, we treated cisplatin-resistant cells with an NF- $\kappa$ B inhibitor while concurrently knocking down VGLL4. Our results revealed that the NF- $\kappa$ B inhibitor restored the increase in IC<sub>50</sub> and stemness induced by si-VGLL4.

## 5. Conclusions

In conclusion, this study demonstrates that VGLL4 enhances cisplatin-resistant sensitivity and suppresses tumor cell

stemness in lung cancer, which is mediated through the regulation of the NF- $\kappa$ B pathway by VGLL4 via COL3A1.

## AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## AUTHOR CONTRIBUTIONS

JJX—Performed material preparation and the experiments. QM—Performed data collection and analysis. WFH—Written the first draft of the manuscript. All authors contributed to the study conception and design. And All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## ACKNOWLEDGMENT

Not applicable.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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