



SND1 confers chemoresistance to cisplatin-induced apoptosis by targeting GAS6-AKT in SKOV3 ovarian cancer cells

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Abstract

Platinum-based (especially cisplatin) chemotherapy is the main treatment after surgery for ovarian cancer. Although the initial treatment is effective, chemotherapy resistance develops rapidly. Therefore, chemotherapy resistance has always been a huge obstacle in the treatment of ovarian cancer. Staphylococcal nuclease domain-containing protein 1 (SND1) is an evolutionarily conserved multifunctional protein that plays a role in promoting tumorigenesis under various stress states. In this study, using MTT and SKOV3 ovarian cancer cells deficient in SND1 were observed to be more apoptotic and to express more apoptotic protein after treatment with cisplatin through the MTT, clone formation, and flow cytometry assays, while cells overexpressing SND1 exhibited a decreased number of apoptotic cells and expression of apoptotic proteins. Moreover, SND1 can regulate the expression of Growth arrest-specific 6 (GAS6) and then activate the AKT signaling pathway to achieve the regulation of sensitivity to cisplatin-induced apoptosis in ovarian cancer.

Keywords SND1 · GAS6 · Chemoresistance · Cisplatin

Background

In ovarian cancer, the combination of optimal tumor surgery and chemotherapy is an important treatment strategy. Chemotherapy drugs for ovarian cancer mainly depend on platinum, especially cisplatin [1, 2]. However, some patients respond well to chemotherapy initially, but later relapse due to multidrug resistance [3, 4]. The mechanisms of cellular resistance to chemotherapeutic drugs are not entirely understood [5]. Staphylococcal nuclease domain-containing protein 1 (SND1; also named p100, Tudor-SN or TDRD11) is a multifunctional protein that has been identified as an

oncoprotein that is implicated in tumorigenesis through diverse mechanisms, including gene transcription [6], pre-mRNA splicing [7, 8], the cell cycle [9, 10], DNA damage repair [11], the programmed cell death degradome [12], adipogenesis [13], and carcinogenesis [14, 15]. It was previously reported that upregulation of SND1 protein is involved in a variety of stimulation processes in vitro and in vivo. SND1 participates in the formation of stress particles and functions in transporting and protecting RNA under external stimuli [16, 17]. SND1 activates the ATM phosphorylation pathway and promotes the process of DNA damage repair [11]. SND1 protein also regulates the long non-coding RNA *UCA1* and promotes chemotherapy resistance of HCC cells [18]. Additionally, SND1 can be located in the endoplasmic reticulum to inhibit HLA processing and participate in the immune escape process [19]. In mice, SND1 is involved in the immune response to chlamydia lung infection [20]. Despite this, the mechanism underlying the effect of SND1 on the cellular apoptosis of SKOV3 cells remains largely unclear.

GAS6 is a secreted protein that interacts with receptor tyrosine kinases to activate downstream signaling pathways and is involved in some biological processes [21]. It has been reported that GAS6 can regulate the sensitivity of lung cancer to chemotherapy drugs through the PI3K/AKT signaling

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pathway [22, 23]. In the present study, we provide evidence regarding the relationship between SND1 and GAS6/AKT, which is also involved in the anti-apoptotic mechanism of SND1 protein in the cisplatin-induced apoptosis of SKOV3 ovarian cancer cells.

Materials and methods

Cell culture

The ovarian cancer cell line SKOV3 was purchased from China Infrastructure of Cell Line Resources (Beijing, China) and maintained in McCoy's 5A medium (M4892; Millipore) containing 10% fetal bovine serum (FBS) at a temperature of 37 °C under 5% CO₂. Stable cell lines were constructed by a previous study [24].

MTT assay and clone formation

Cell proliferation was detected by MTT following the manufacturer's protocol every 24 h. And, cells were seeded in a fresh 6-well plate and maintained in media with 10% FBS, replacing the medium every 4 days for the colony formation assay. After 14 days, cells were fixed and stained with 0.1% crystal violet (Sigma-Aldrich), and clones were counted and analyzed.

Apoptosis assay

Cells were stimulated and collected using 5 µg/mL or 10 µg/mL cisplatin (P4394, Sigma-Aldrich). Cell apoptosis was detected by the Annexin V-FITC Apoptosis Detection Kit (AD10, Dojindo Laboratories), according to the manufacturer's protocol. Flow cytometry was used to analyze the percentage of FITC-positive apoptotic cells (BD Biosciences FACSVerse, Becton Dickinson).

Western blot and antibodies

Total cell lysates were harvested in RIPA lysis buffer (R0010; Solarbio) supplemented with a proteinase inhibitor cocktail (4693159001; Roche). Equal cell lysates were separated in 8 or 10% SDS-PAGE gel and tested using specific antibodies: anti-Cleaved PARP (9541s; Cell Signaling Technology), anti-Cleaved caspase-3 (9661s; Cell Signaling Technology), anti-GAS6 (67202S; Cell Signaling Technology), anti-AKT (4685s; Cell Signaling Technology), anti-p-AKT (4060s; Cell Signaling Technology), and anti-beta-actin (A1978; Millipore Sigma). Anti-SND1 antibody was generated against the amino acids 507–674 of SND1 by the Institute of Medical Technology, University of Tampere, Tampere, Finland [25].

RNA extraction and quantitative PCR

Total RNA was isolated using Trizol reagent (15596026; Ambion), and according to the manufacturers' protocol, reverse transcription was performed with a Revert Aid First-Strand cDNA Synthesis Kit (F-479L; Thermo Fisher Scientific). The quantitative PCR (qPCR) primer sequences are described in Table 1. SYBR Green Master Mix (4913850001; Roche) was used for qPCR detection. The relative mRNA fold changes were calculated using the 2-delta delta Ct method. GAPDH was used as a reference gene.

Statistical analysis

Each experiment was conducted independently at least three times and data are presented as the means ± SEM. Values of $P < 0.05$ were considered to indicate statistical significance. IBM SPSS Statistics 20 Software was used for the above statistical analysis.

Table 1 The quantitative PCR primer sequences used in this study

Gene symbol	Forward primer	Reverse primer
<i>SND1</i>	GGTGGACTACATTAGACCAGCC	AGACCTTTGCTGACAAGAGCCTC
<i>GAS6</i>	CCTTCCATGAGAAGGACCTCGT	GAAGCACTGCATCCTCGTGT
<i>AKT1</i>	TGGACTACCTGCACTCGGAGA	GTGCCGCAAAAAGGTCTTCATGG
<i>AKT3</i>	CGAACCAAAAATAACTTTCCC	AAACAGAACGACCAAAAGCCAAAAC
<i>BIRC1</i>	CCGAACAGGAAGTCTTCTCAC	CCACAGACAGTTCTTTCAGGCAC
<i>GADD45</i>	CTGGAGGAAGTGCTCAGCAAAG	AGAGCCACATCTCTGTCGTCGT
<i>Bcl-2</i>	TTGCCAGCCGGAACCTATG	CGAAGGCGACCAGCAATGATA
<i>GAPDH</i>	GTCTCCTCTGACTTCAACAGCG	ACCACCTGTTGCTGTAGCCAA
<i>SFRP1</i>	CAATACCACGGAAGCCTTAAGC	GCAAACCTCGCTTGCACAGAGATG
<i>INHBA</i>	GGATGACATTGGAAGGAGGGCA	ACTGACAGGTCACTGCCTTCT
<i>CDK4</i>	GGGGACCTAGAGCAACTTACT	CAGCGCAGTCCTTCCAAAT

Results

SND1 protects the viability of SKOV3 cells from cisplatin treatment

The major treatment strategy for ovarian cancer is surgery and chemotherapy, and most chemotherapy agents are based on platinum. Therefore, here, we observed the effects of SND1 on sensitivity to cisplatin in the ovarian cancer cell line, SKOV3. Previously, we stably knocked down SND1 in SKOV3 cells with shRNA lentivirus (sh1 and sh2) and stably overexpressed SND1 in cells with a Flag tag (Flag-SND1) in the SKOV3 cell line.

We first tested different concentrations of cisplatin to determine the cytotoxicity of the SKOV3 cells by MTT assay and found that the IC50 was approximately 10 µg/mL after 24 h and 5 µg/mL after 72 h of treatment (Fig. 1A). We found that the viability of the SKOV3 cells was significantly inhibited by cisplatin in a time- and concentration-dependent manner.

To test whether SND1 increases the sensitivity of SKOV3 cells to cisplatin, we examined the SKOV3 cell viability through the clone formation assay. SKOV3 cells were seeded in 6-well plates at a density of 2000 cells/well

and cultured with 5 µg/mL cisplatin for 72 h, and the clone number was counted after culturing for 2 weeks. We found that the viability of SKOV3 cells was slightly inhibited by SND1 knockdown, especially under the cisplatin treatment (Fig. 1B and C). The cell viability increased when SND1 was overexpressed in SKOV3 cells even though with the cisplatin treatment (Fig. 1D and E). These data suggest that SND1 may play a role in resistance to cisplatin chemotherapy.

SND1 suppresses cisplatin-induced apoptosis of SKOV3 cells

Then, we detected cell apoptosis using flow cytometry. The knockdown and overexpression stable cells were treated with two concentrations of cisplatin (5 µg/mL and 10 µg/mL) for 72 h, and cell survival was determined by apoptosis assay. Apoptosis (UR quadrant) in high doses of cisplatin (10 µg/mL) was more serious than that in low doses (5 µg/mL) in all kinds of stable SKOV3 cells. SND1-deficient cells, both sh1 and sh2, displayed more sensitivity to cisplatin than control cells (pLKO), especially at high concentrations. Only 1.97% of cells encountered apoptosis in normal pLKO, and the values of apoptosis slightly increased to 4.53% and 4.42%, respectively, after treatment with cisplatin at 5 µg/

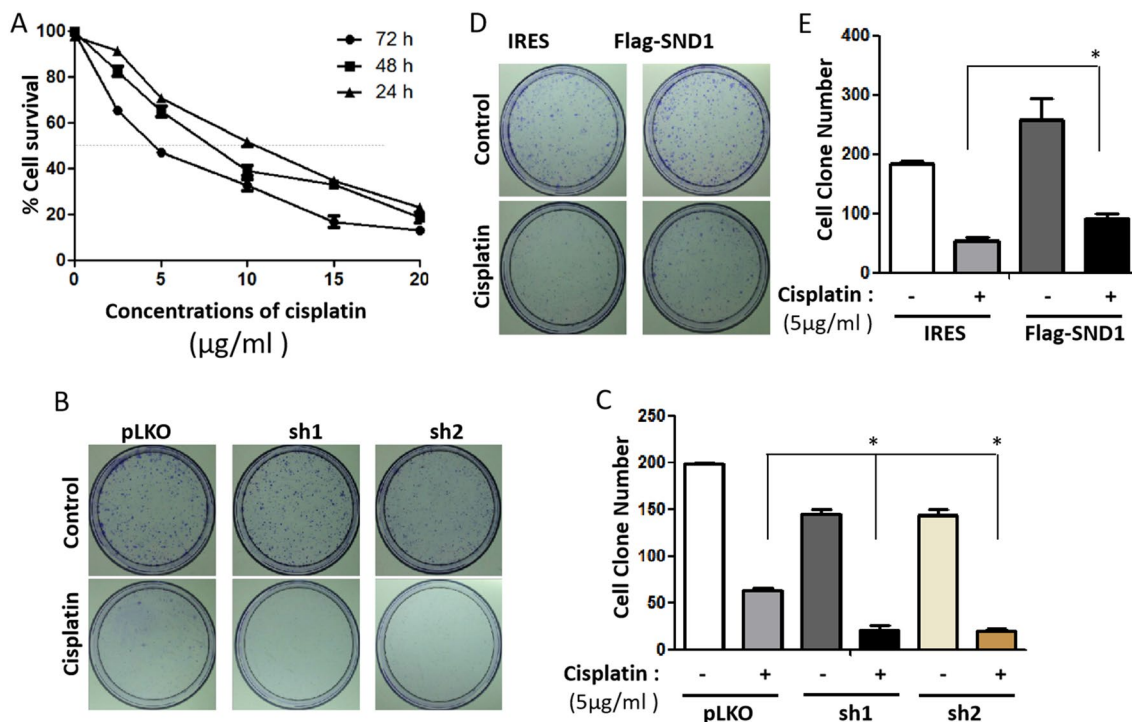


Fig. 1 SND1 protects the viability of the SKOV3 cells from cisplatin treatment. **A** SKOV3 cells were treated with various concentrations of cisplatin for 24, 48, and 72 h and then cell survival was determined by the MTT assay. Clone formation assays transfected with nega-

tive control (pLKO), SND1-shRNA, and Flag-SND1 (**B** and **D**) and summary of the clone number (**C** and **E**). All data are based on three independent experiments and presented as the mean ± SEM followed by Student's *t* test, **P* < 0.05

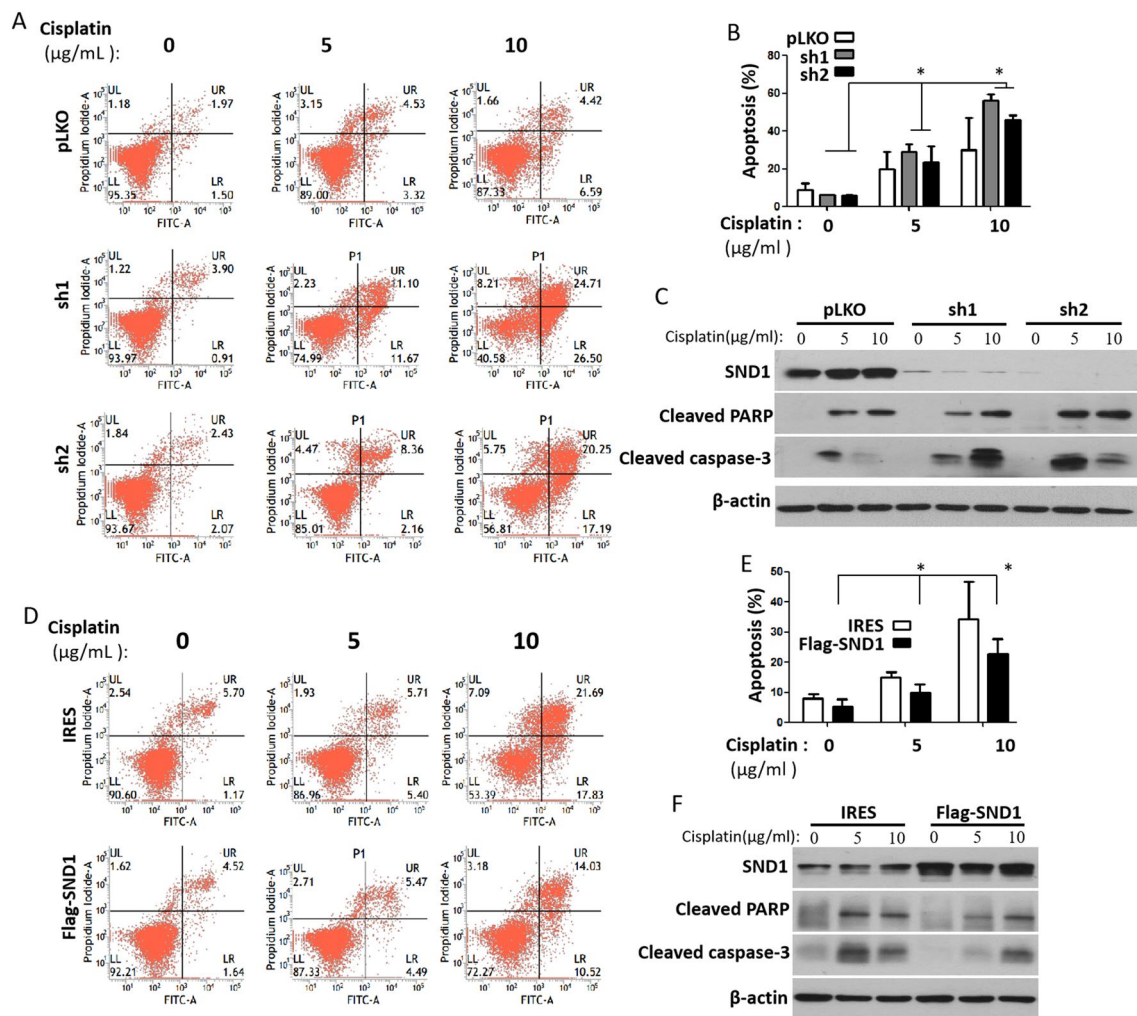


Fig. 2 SND1 suppresses cisplatin-induced apoptosis of SKOV3 cells. Flow cytometry for apoptosis assay was performed by Annexin V-FITC/propidium iodide stain in SND1-shRNA (A and B) and Flag-SND1 (D and E) cells with 0, 5, and 10 μg/mL cisplatin treatment. Independent sample Student's *t* test (**P* < 0.05) was performed. C

mL and 10 μg/mL (Fig. 2A and B). SND1 overexpression cells showed greater resistance to cisplatin than control cells (IRES) (Fig. 2D and E). The effect on apoptosis was confirmed by testing the expression status of apoptotic proteins, such as caspase-3 and cleaved PARP. The results showed that cisplatin treatment alone slightly increased the level of apoptotic proteins in SKOV3 cells, while knockdown of SND1 caused markedly increased expression of active caspase-3 and formation of cleaved PARP (Fig. 2C). We also determined the expression of apoptotic proteins in SND1 overexpression cells. Both cleaved PARP and cleaved caspase-3 were increased after treatment with cisplatin, while overexpression of SND1 could suppress cisplatin-induced apoptotic protein expression (Fig. 2F). These data indicate that the enhancement of cisplatin resistance in SKOV3 cells

and F, SKOV3 cells with stable depletion of SND1 by shRNA and SKOV3 cells stably expressing SND1-FLAG were collected, followed by Western blot using antibodies against SND1, cleaved PARP and cleaved caspase-3, or anti-β-actin antibody as the control. pLKO and IRES cell lines were used as controls

by SND1 may be mediated through the induction of apoptosis via a caspase-dependent mechanism and by suppression of the cisplatin-induced apoptotic protein expression.

The GAS6/AKT signaling pathway was involved in the resistance of SND1 to cisplatin

To understand the molecular mechanism of the chemoresistance effect of SND1 in cisplatin-induced SKOV3 apoptosis, we screened our previous expression profile chip in SKOV3 cells with stable knockdown of SND1. We found that knockdown of SND1 affected the expression of more than a dozen genes related to chemotherapy sensitivity, including AKT, GAS6, SFRP1, and BCL2. Real-time quantitative PCR was used to confirm the

correlation between SND1 and the expression of potential genes in two shRNA knockdown cells and overexpression cells (Fig. 3A and B). The results showed that only the expression of GAS6 was the same as that of SND1 in both knockdown and overexpression cells. However, the expression trends of other genes such as AKT and BCL2 are not completely consistent with SND1.

Next, we examined whether SND1 and GAS6 expressions were significantly decreased under cisplatin treatment (Fig. 4A and B). While the cisplatin-induced decrease in GAS6 expression was much more serious in SND1-down-regulated cells, it was somewhat relief in SND1-overexpressing cells. Furthermore, western blotting results showed the same protein expression pattern between SND1 and GAS6 under cisplatin treatment. Previous studies have proven that GAS6 can activate the phosphorylation of AKT during chemotherapy, especially cisplatin. Therefore, we detected AKT and p-AKT in our model. Total AKT was consistently stably expressed upon treatment with cisplatin when SND1 expression in SKOV3 cells was downregulated or upregulated. However, p-AKT was severely decreased when SND1 was depleted (Fig. 4C, lanes 1, 4, and 7) and that was significantly increased in SND1-upregulated cells (Fig. 4D, lanes 1 and 4). Cisplatin treatment drastically enhanced this expression type (Fig. 4C, lanes 2–3, 5–6, and 8–9; D, lanes 2–3 and 5–6). These data suggest that SND1 may be involved in

resistance to cisplatin-induced apoptosis by regulating the GAS6/AKT signaling pathway.

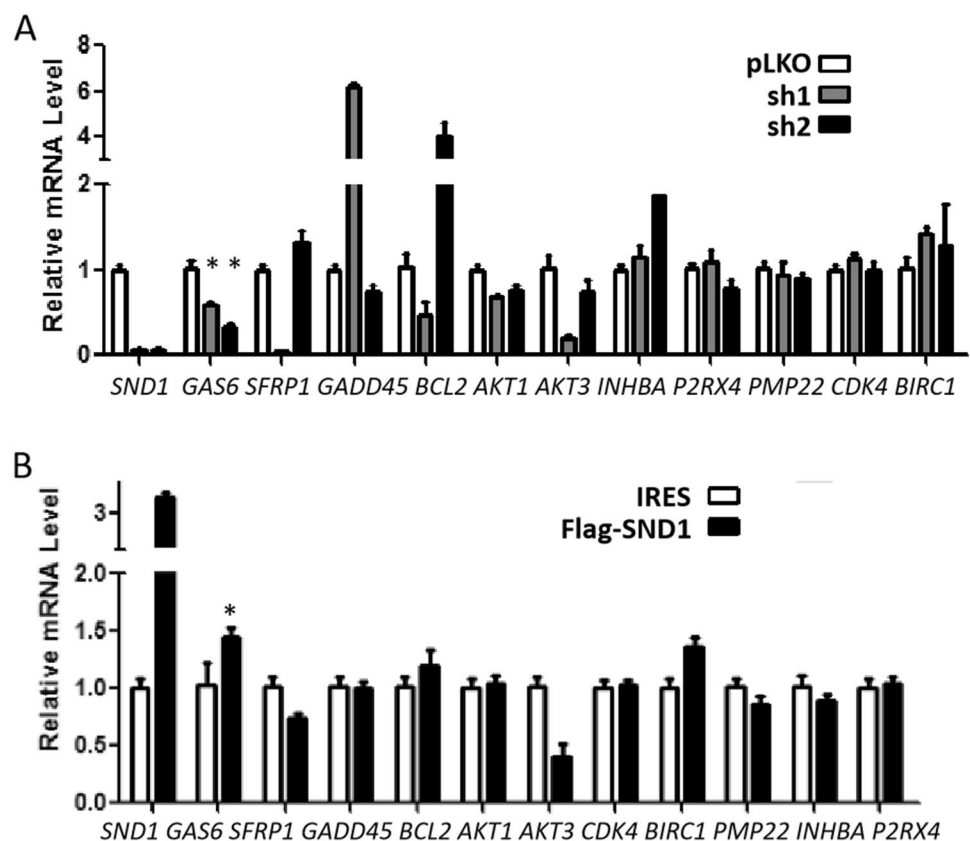
Discussion

As a transcription coactivator, SND1 is highly expressed in ovarian cancer cells and activates SLUG transcription, which inhibits CDH1, thereby promoting the process of EMT and ultimately leading to metastasis of ovarian cancer [24]. In recent years, an increasing number of studies have found that EMT not only regulates tumorigenesis and metastasis but also has a close relationship with chemotherapy drug resistance and recurrence of tumor cells [26].

In the present study, we demonstrated that SND1 is involved in cisplatin-induced apoptosis in SKOV3 cells. After cisplatin treatment, cell apoptosis increased in SND1-knockdown cells, while it decreased in cells overexpressing SND1. These results indicated that SND1 inhibited the chemotherapy sensitivity in SKOV3 cells (Fig. 4E).

Mechanistically, we analyzed the expression profile chip results of SND1-shRNA in SKOV3 cells through genes related to apoptosis and chemotherapy sensitivity and further found that its functions regulate the potential mechanism. SND1 may promote the expression of GAS6, which may regulate the chemotherapy sensitivity of SKOV3 cells

Fig. 3 Screened chemotherapy sensitivity-related genes from expression profile chip in SKOV3 cells of stably knock-down SND1. **A, B** 11 chemotherapy sensitivity-related genes were measured by qPCR in SND1-knockdown cells (sh1 and sh2) and expressing Flag-SND1 cells. GAPDH was used as a reference gene. Independent sample Student's *t* test (**P* < 0.05) were performed



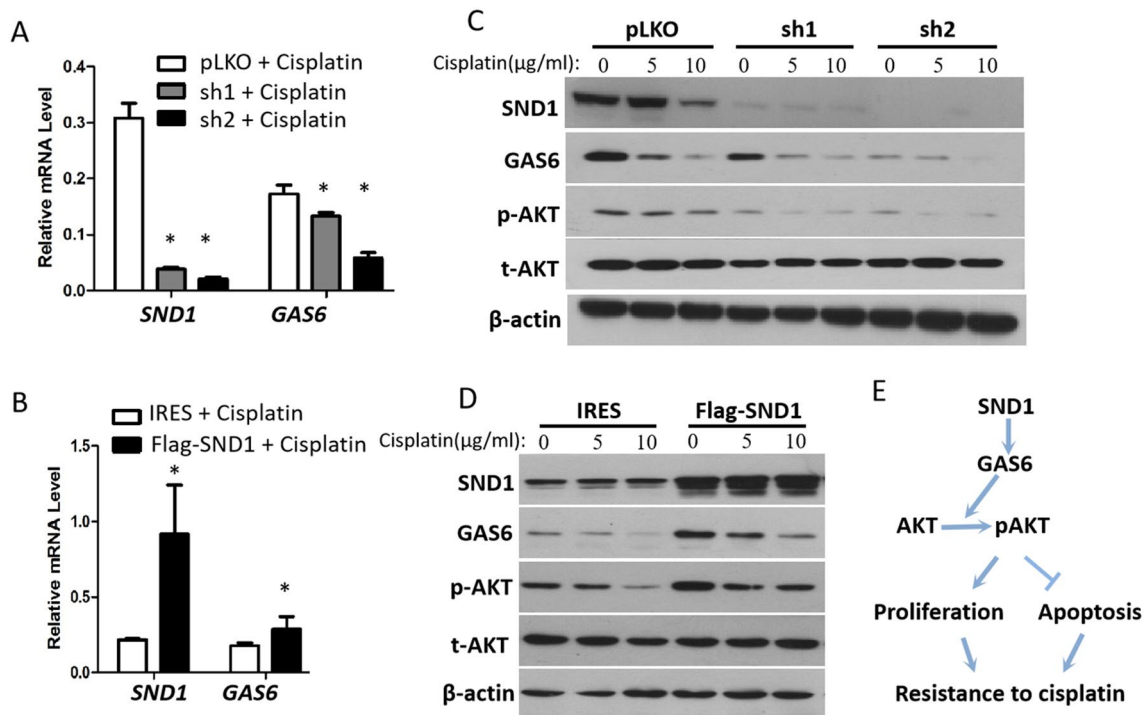


Fig. 4 GAS6/AKT signaling pathway was involved in the resistance of SND1 to cisplatin. **A, B** GAS6 mRNA expression was examined by qPCR in SND1-knockdown cells (sh1 and sh2) and expressing Flag-SND1 cells with 10 $\mu\text{g}/\text{mL}$ cisplatin treatment for 72 h. Student's *t* test ($*P < 0.05$) was performed. **C, D** shRNA cells and Flag-SND1 cells under 0, 5, and 10 $\mu\text{g}/\text{mL}$ cisplatin treatment were har-

vested followed by Western blot with antibodies against GAS6, total AKT (t-AKT), and phosphorylated AKT (p-AKT) and β -actin as the control. **E** Proposed model of SND1 as a novel transcriptional coactivator of GAS6 expression in promoting the resistance of cisplatin in SKOV3 cells

through the AKT signaling pathway. This is consistent with previous reports that GAS6 binds to the receptor Axl [27], which leads to the autophosphorylation of Axl and then binds to the growth factor receptor-binding protein, thereby activating the PI3K/AKT signaling pathway, enhancing the survival and invasion of cells and inhibiting apoptosis [22, 28]. However, whether transcriptional activation by SND1 is the only mechanism to maintain GAS6 expression in SKOV3 cells or other transcription factors or epigenetic regulation mechanisms are also involved needs further investigation.

The above studies indicate that SND1 may regulate GAS6 transcriptional activity and is involved in the sensitivity of tumor cells to chemotherapy drugs through the AKT signaling pathway. Therefore, the in-depth study of this mechanism not only provides us with the basis for the occurrence and development of diseases but also provides the possibility for the treatment of diseases.

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Author contributions JY and LX contributed to the study conception and design. Material preparation, data collection, and analysis were performed by CH, LH, and YR. The first draft of the manuscript was written by LX and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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