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ORIGINAL ARTICLE

Hsa_circ_0096157 silencing suppresses autophagy and reduces cisplatin resistance in non-small cell lung cancer by weakening the Nrf2/ARE signaling pathway

Huasong Lu[1] · Jinliang Kong[1] · Shuangqi Cai[1] · Hong Huang[1] · Jing Luo[1] · Lihua Liu[1]

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Abstract

Background Non-small cell lung cancer (NSCLC) is the leading cause of cancer morbidity and mortality worldwide, and new diagnostic markers are urgently needed. We aimed to investigate the mechanism by which hsa_circ_0096157 regulates autophagy and cisplatin (DDP) resistance in NSCLC.

Methods A549 cells were treated with DDP (0 μg/mL or 3 μg/mL). Then, the autophagy activator rapamycin (200 nm) was applied to the A549/DDP cells. Moreover, hsa_circ_0096157 and Nrf2 were knocked down, and Nrf2 was overexpressed in A549/DDP cells. The expression of Hsa_circ_0096157, the Nrf2/ARE pathway-related factors Nrf2, HO-1, and NQO1, and the autophagy-related factors LC3, Beclin-1, and p62 was evaluated by qRT−PCR or western blotting. Autophagosomes were detected through TEM. An MTS assay was utilized to measure cell proliferation. The associated miRNA levels were also tested by qRT−PCR.

Results DDP (3 μg/mL) promoted hsa_circ_0096157, LC3 II/I, and Beclin-1 expression and decreased p62 expression. Knocking down hsa_circ_0096157 resulted in the downregulation of LC3 II/I and Beclin-1 expression, upregulation of p62 expression, and decreased proliferation. Rapamycin reversed the effect of interfering with hsa_circ_0096157. Keap1 expression was lower, and Nrf2, HO-1, and NQO1 expression was greater in the A549/DDP group than in the A549 group. HO-1 expression was repressed after Nrf2 interference. In addition, activation of the Nrf2/ARE pathway promoted autophagy in A549/DDP cells. Moreover, hsa_circ_0096157 activated the Nrf2/ARE pathway. The silencing of hsa_circ_0096157 reduced Nrf2 expression by releasing miR-142-5p or miR-548n. Finally, we found that hsa_circ_0096157 promoted A549/DDP cell autophagy by activating the Nrf2/ARE pathway.

Conclusion Knockdown of hsa_circ_0096157 inhibits autophagy and DDP resistance in NSCLC cells by downregulating the Nrf2/ARE signaling pathway.

Keywords Hsa_circ_0096157 · Nrf2/ARE signaling pathway · Autophagy · Cisplatin · Non-small cell lung cancer

Introduction

Non-small cell lung cancer (NSCLC) is the most common type of cancer in the world, with an approximately 20% 5-year survival rate [1]. In nearly two-thirds of patients with NSCLC, the disease has already advanced locally or metastatically by the time they are diagnosed, and many patients with early-stage disease eventually develop metastatic recurrence [2]. The prognostic variables include tumor-node-metastasis (TNM) stage [3]. Surgery, radiotherapy, chemotherapy, immunotherapy, or molecularly targeted therapy are applied to treat NSCLC [4]. However, chemotherapy resistance limits the efficacy of treatment for NSCLC and leads to a poor prognosis [5]. In addition, the poor prognosis of NSCLC patients is primarily associated with a patient being diagnosed at an advanced stage of disease [6]. As a result, new diagnostic markers and treatments are urgently needed.

Circular RNAs (circRNAs), a novel class of noncoding RNAs, are widely found in eukaryotic cytoplasms with highly stable closed-loop structures [7]. Studies have shown that circRNAs participate in cell proliferation, metastasis,

✉ Lihua Liu
lhlhliu@163.com

[1] Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital of Guangxi Medical University, No. 6, Shuangyong Road, Nanning 530021, People's Republic of China

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and drug resistance [8]. Furthermore, circRNAs play a vital regulatory role in various human malignancies, including NSCLC [9]. CircRNAs were found to participate in chemotherapy resistance in lung cancer. For example, Li et al. reported that hsa_circ_0068252 is involved in cisplatin (DDP) resistance through the miR-1304-5p/PD-L1 axis in NSCLC [10]. Hao et al. revealed that circ_0110498 promoted DDP resistance in NSCLC through mediating the miR-1287-5p/RBBP4 axis [11]. Additionally, studies have shown that hsa_circ_0096157 promoteed DDP resistance by promoting NSCLC cell proliferation and inhibiting apoptosis [12]. However, the specific mechanism of hsa_circ_0096157 and DDP resistance in NSCLC is unclear.

The Nrf2/antioxidant response element (ARE) signaling pathway is a central defense mechanism against oxidative stress and is crucial for the development and progression of many diseases [13]. Nrf2 contributes to anti-inflammatory effects by regulating inflammatory cell recruitment and gene expression via the ARE [14]. The Nrf2 signaling pathway is highly mutationally activated in NSCLC and promotes the malignant progression of lung cancer through multiple mechanisms [15]. Wang et al. reported that circKEAP1 inhibited lung adenocarcinoma through the miR-141-3p/KEAP1/NRF2 axis [16]. Autophagy is a highly conserved and regulated intracellular lysosomal degradation pathway essential for cell survival [17]. In mammals, the Nrf2/ARE pathway and autophagy are the central intracellular defense systems against antioxidant damage and maintenance of homeostasis [18]. However, it is unclear whether hsa_circ_0096157 acts on the Nrf2/ARE signaling pathway to affect autophagy and DDP resistance in NSCLC.

Based on the above background, this paper explored whether the mechanism by which hsa_circ_0096157 regulates autophagy and DDP resistance involves the Nrf2/ARE signaling pathway through in vitro cellular experiments. Our study provides important clues for a deeper understanding of the molecular mechanisms underlying the development of DDP resistance in NSCLC and for identifying new therapeutic targets.

Materials and methods

Cell culture and treatment

NSCLC A549 (CC0202) and DDP-resistant A549/DDP cells were obtained from CELLCOOK (Guangzhou, China). The cells were cultured in F12K media supplemented with 2.5 g/L $NaHCO_3$ and 10% FBS. DDP (0 μg/mL or 3 μg/mL) was applied to A549 and A549/DDP cells for 24 h and 48 h. To study the role of hsa_circ_0096157 in autophagy in A549/DDP cells, 200 nM rapamycin, an autophagy activator, was added.

Cell transfection

Hsa_circ_0096157 and Nrf2 were knocked down in A549/DDP cells, and siRNAs and miR-142-5p or miR-548n inhibitors were synthesized by GenePharma (Shanghai, China). The sequence of si-NC (negative control) was 5′-GAGGGG GAGAUAU-3′; the sequence of Nrf2-siRNA-1 was 5′-GGA AAGACAAGAACAACTCCA-3′; and the sequence of Nrf2-siRNA-2 was 5′-UAAUUGUCAACUACUGUCAGUU-3′. Concurrently, Nrf2 was overexpressed in A549/DDP cells, and the DNA sequence encoding Nrf2 was amplified and ligated to the pcDNA3.1 plasmid. siRNAs or overexpression plasmids were transfected into A549/DDP cells for 48 h with Lipofectamine™ 2000.

Cell proliferation

The proliferation rate of NSCLC cells was analyzed using MTS (G5421, Promega Biotech). Briefly, NSCLC cells treated as described above were inoculated in 96-well plates (100 μL/well). The MTS/PMS mixture (20 μL) was added to NSCLC cells for 2 h at 37 °C. Finally, the plates were read at 492 nm to determine NSCLC cell proliferation.

Quantitative real-time PCR (qRT−PCR)

Total RNA was extracted from NSCLC cells. cDNA was reverse transcribed with a Prime Script RT Master Mix kit (RR036A, Takara). Hsa_circ_0096157, Keap1, Nrf2, HO-1, NQO1, and miRNA expression was detected using a 2× EasyTaq PCR SuperMix kit (AS111-03, TransGen Biotech). U6 was used as the miRNA internal control, and GAPDH was used as a control for the other RNAs.

Western blot

Total proteins were extracted from NSCLC cells using RIPA buffer (P0013B, Beyotime). Proteins were boiled at 100 °C, separated by SDS−PAGE, and transferred to PVDF membranes. After 2 h of blocking, the proteins were incubated overnight with primary antibodies at 4 °C. Then, the proteins were incubated with HRP-labeled secondary antibodies for 1 hour. An enhanced chemiluminescence (ECL) solution was used for color development. GAPDH was used as an internal reference. The primary antibodies used were against LC3 (Proteintech, 14600-1-AP, 1:2000), Beclin-1 (Proteintech, 11306-1-AP, 1:2000), p62 (Proteintech, 18420-1-AP, 1:2000), S6K1 (Proteintech, 14485-1-AP, 1:2000), p-S6K1 (Proteintech, 28735-1-AP, 1:2000), Keap1 (Proteintech, 80744-1-RR, 1:2000), Nrf2 (Proteintech, 80593-1-RR,

1:2000), HO-1 (Proteintech, 10701-1-AP, 1:2000), NQO1 (Proteintech, 11451-1-AP, 1:2000) and GAPDH (Proteintech, 60004-1-Ig, 1:5000).

Transmission electron microscopy (TEM)

A549 and A549/DDP cells were digested and washed with precooled PBS, and the supernatant was discarded after centrifugation for 5 min at 1500 rpm. The cells were collected in 1.5 mL EP tubes. Fixation and embedding were performed at 4 °C. for 24 h; the cells were stained with 1% OsO_4 after being fixed with 2.5% glutaraldehyde. We stained autophagosomes with 3% lead citrate-uranyl acetate and photographed them with a JEM-1100 transmission electron microscope (JEOL, Japan) after they were dehydrated with alcohol.

Construction of the hsa_circ_0096157-miRNA-Nrf2 network

The miRNA binding sites of hsa_circ_0096157 and Nrf2 were predicted using miRanda and TargetFinder software, and the screened miRNAs were used to construct a circRNA−miRNA−mRNA ceRNA interaction network.

Statistical analysis

All experiments were independently repeated three times. The experimental data were analyzed with SPSS 20.0 and GraphPad Prism 9 and are expressed as the mean ± standard deviation. Student's *t* tests were applied for comparisons between two groups. ANOVA was performed for comparisons between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Determination of DDP concentration in A549 cells

To explore cell resistance to DDP, we first screened the optimal concentration of DDP for treatment. A549 cells were treated with 0, 1, 3, 10, or 30 μg/mL DDP for 48 h, and the MTS data revealed that there was no significant change in cell viability after treatment with 1 μg/mL DDP;

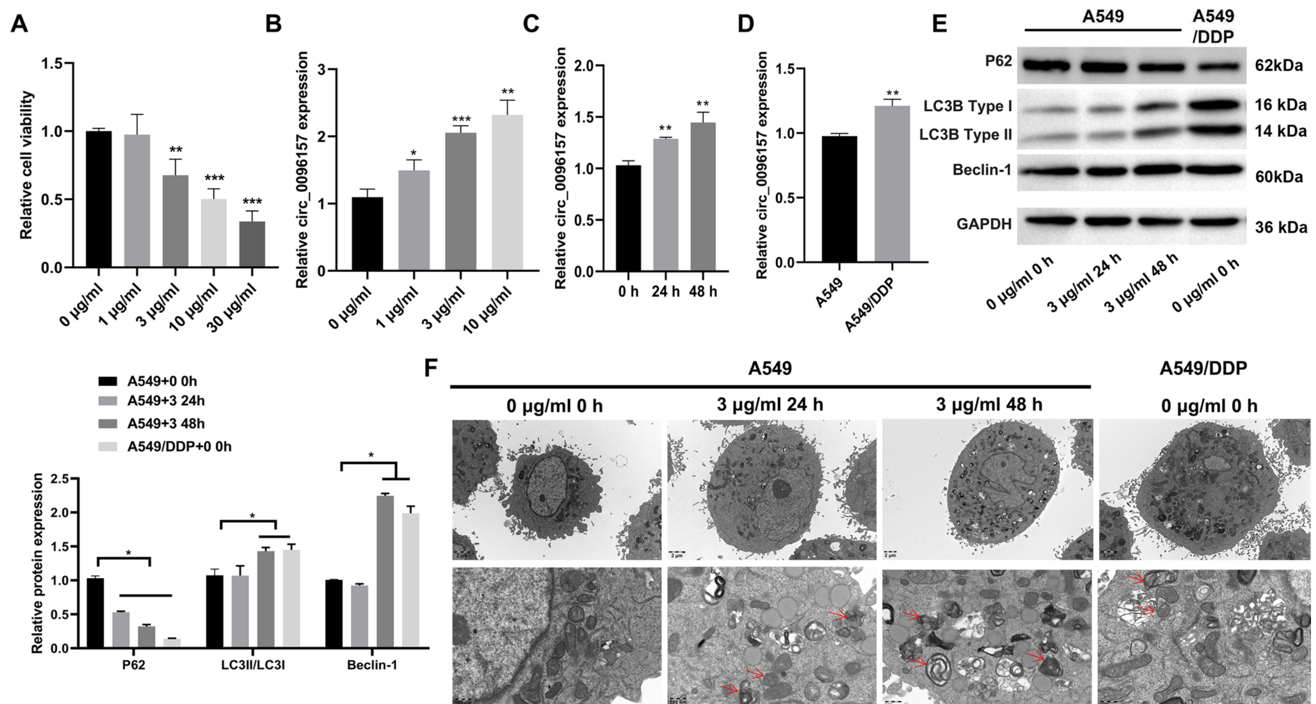

Fig. 1 Hsa_circ_0096157 expression increased in DDP-treated A549 cells and A549/DDP cells. **A** A549 cells were treated with 0, 1, 3, 10, or 30 μg/mL DDP for 48 h, and cell viability was determined using MTS. **B** Hsa_circ_0096157 expression was analyzed via qRT−PCR in A549 cells treated with 0, 1, 3, and 10 μg/mL DDP for 48 h. **C** A549 cells were treated with DDP (3 μg/mL) for 24 h and 48 h, and hsa_circ_0096157 expression was assessed via qRT−PCR. **D** The expression of hsa_circ_0096157 in A549 and A549/DDP cells was assessed via qRT−PCR. **E** Western blotting was utilized to evaluate the levels of autophagy-related factors (LC3, Beclin-1, and p62). **F** TEM measurement of autophagosomes. The quantitative data were obtained from three independent replicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

after treatment with 3 μg/mL DDP, there was a clear trend toward decreased cell viability compared with that of the control group (Fig. 1A). Moreover, DDP increased hsa_circ_0096157 expression, and the increase in the level of hsa_circ_0096157 increased in a dose-dependent manner with increasing concentrations of DDP, especially 3 μg/mL DDP (Fig. 1B). In summary, we chose 3 μg/mL DDP as the optimal concentration for the treatment of A549 cells.

Hsa_circ_0096157 expression increased in DDP-treated A549 cells and A549/DDP cells

First, A549 cells were treated with DDP (3 μg/mL) for 24 h and 48 h. Hsa_circ_0096157 expression was enhanced in A549 cells treated with 3 μg/mL DDP. As the processing time increased, hsa_circ_0096157 expression also increased (Fig. 1C). Additionally, compared with A549 cells, A549/DDP cells had higher hsa_circ_0096157 expression (Fig. 1D). We then examined autophagy in A549 and A549/DDP cells. Compared with A549 cells treated with 0 μg/mL DDP, A549 cells treated with 3 μg/mL DDP exhibited decreased p62 levels and increased LC3 II/I and Beclin expression, especially at 48 h. Compared with A549 cells, A549/DDP cells had lower p62 expression but higher LC3 II/I and Beclin expression (Fig. 1E). The TEM results showed that DDP increased mitophagy in the A549-sensitive cells. DDP resistance increased mitochondrial autophagy in A549 cells (Fig. 1F). Therefore, cells were treated with 3 μg/mL DDP for 48 h for subsequent study.

Knockdown of hsa_circ_0096157 decreased autophagy and inhibited DDP resistance in A549/DDP cells

Next, we interfered with hsa_circ_0096157 expression. Figure 2A showed that si-hsa_circ_0096157 significantly reduced hsa_circ_0096157 expression, indicating that we successfully transfected cells with the interfering fragment hsa_circ_0096157. Therefore, we next studied the effects of hsa_circ_0096157 on autophagy and DDP resistance in A549/DDP cells by transfecting fragments that interfered with hsa_circ_0096157 and treating them with DDP. Compared with those in the si-NC group, LC3 II/I and Beclin-1 expression was upregulated, and p62 expression was downregulated in the si-NC+3 μg/mL DDP group. After interference with hsa_circ_0096157, LC3 II/I and Beclin-1 expression was downregulated, and p62 expression was upregulated (Fig. 2B). In addition, compared with that in the si-NC group, A549/DDP cell proliferation was reduced in the si-NC+3 μg/mL DDP group, and interference with hsa_circ_0096157 further inhibited proliferation (Fig. 2C).

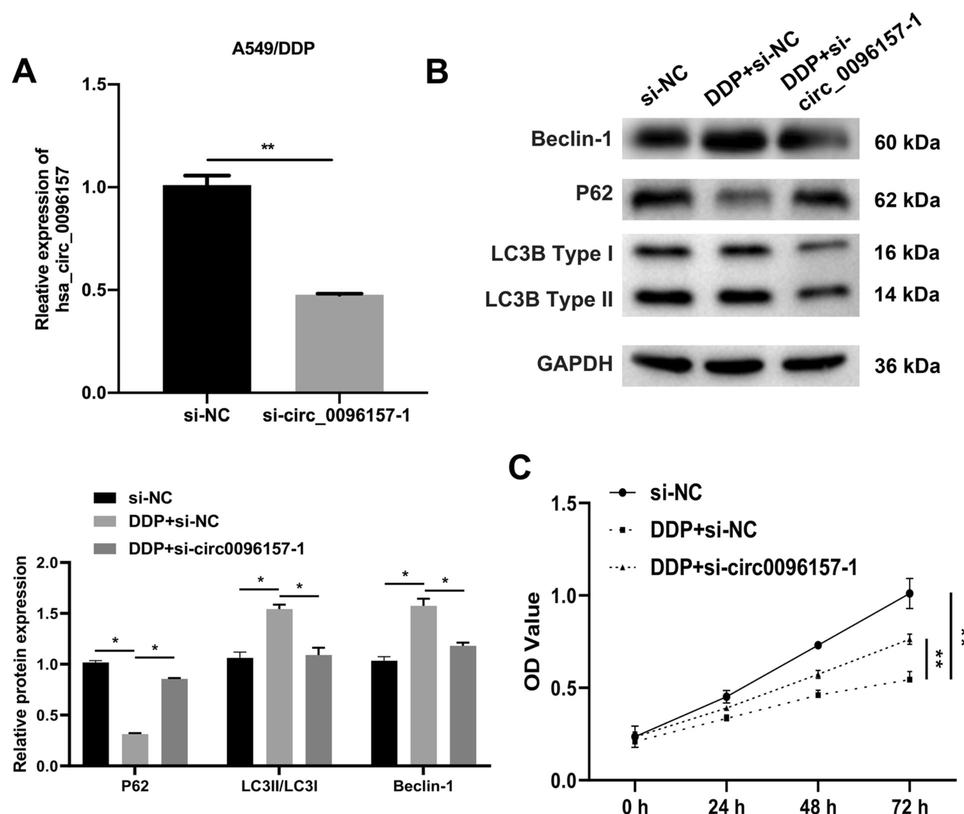

Fig. 2 Knockdown of hsa_circ_0096157 decreased autophagy in A549/DDP cells and inhibited DDP resistance. **A** The interference efficiency of hsa_circ_0096157 was assessed through qRT−PCR. **B** After the transfection of fragments containing hsa_circ_0096157 and DDP, Western blotting was performed to evaluate the expression of the autophagy-related factors LC3, Beclin-1, and p62 in A549/DDP cells. **C** Proliferation of A549/DDP cells was monitored through the MTS assay. The quantitative data were obtained from three independent replicate experiments. *$P<0.05$, **$P<0.01$

Overall, interference with hsa_circ_0096157 decreased autophagy in A549/DDP cells and inhibited DDP resistance.

The autophagy activator rapamycin reversed the effect of hsa_circ_0096157 knockdown on autophagy and DDP resistance in A549/DDP cells

Next, to further investigate the effect of hsa_circ_0096157 on autophagy in A549/DDP cells, we added 200 nM rapamycin, an mTOR inhibitor. Compared with that in the si-NC+3 μg/mL DDP group, hsa_circ_0096157 expression in the si-hsa_circ_0096157+3 μg/mL DDP group decreased. The addition of rapamycin promoted hsa_circ_0096157 expression compared with that in the si-hsa_circ_009615Nrf2, HO-1, and NQO1 expression in A549NQO1 and HO-1 expression was asseNrf2. E. After Nrf2 int7+3 μg/mL DDP group (Fig. 3A). In addition, after interference with hsa_circ_0096157, LC3 II/I and Beclin-1 expression was downregulated, and p-S6K1 and p62 expression were upregulated in A549/DDP cells; rapamycin upregulated LC3 II/I and Beclin-1 and downregulated p-S6K1 and p62 mediated by hsa_circ_0096157 silencing in A549/DDP cells (Fig. 3B). Moreover, the TEM results revealed that hsa_circ_0096157 silencing decreased the number of autophagosomes in A549/DDP cells, while rapamycin increased the number of autophagosomes in A549/DDP cells after hsa_circ_0096157 silencing (Fig. 3C). In addition, A549/DDP cell proliferation was repressed after interference with hsa_circ_0096157. After the addition of rapamycin, proliferation increased (Fig. 3D). Moreover, A549/DDP cells were treated with an autophagy activator (metformin) after hsa_circ_0096157 silencing. Western blot analysis revealed that hsa_circ_0096157 silencing downregulated LC3 II/I and Beclin-1 and upregulated p62 in A549/DDP cells, and these effects were reversed by metformin (Fig. S1A). Hsa_circ_0096157 silencing also inhibited the proliferation of A549/DDP cells, and metformin enhanced the proliferation of A549/DDP cells, which was mediated by hsa_circ_0096157 silencing (Fig. S1B). These results indicated that the knockdown of hsa_circ_0096157 attenuated DDP resistance in A549/DDP cells by regulating the autophagy pathway.

Nrf2/ARE pathway was activated in A549/DDP cells

Next, we assessed the expression of Nrf2/ARE signaling pathway-related factors (Keap1, Nrf2, HO-1, and NQO1) in A549 and A549/DDP cells. Moreover, compared with those in A549 cells, Keap1 expression was decreased, and Nrf2, HO-1, and NQO1 expression was increased in A549/DDP cells (Fig. 4A, B). In addition, the data revealed

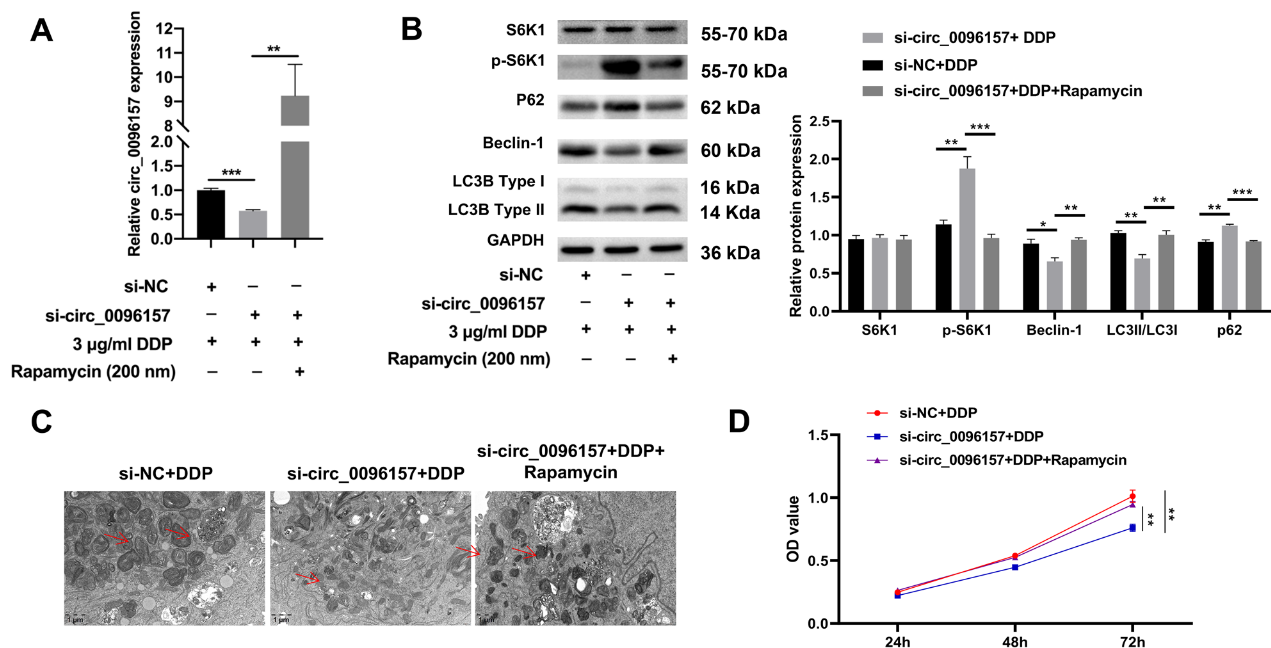

Fig. 3 The autophagy activator rapamycin reversed the effect of hsa_circ_0096157 knockdown on autophagy and DDP resistance in A549/DDP cells. After transfection of the fragments that interfered with hsa_circ_0096157 via simultaneous treatment with DDP and then combined treatment with the autophagy activator rapamycin, **A** qRT−PCR was performed to monitor hsa_circ_0096157 expression. **B** Western blot analysis of S6K1, p-S6K1 and autophagy-associated factor (LC3, Beclin-1, and p62) expression in A549/DDP cells. **C** TEM image of autophagosomes in A549/DDP cells. **D** Proliferation of A549/DDP cells was monitored via the MTS assay. The quantitative data were obtained from three independent replicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

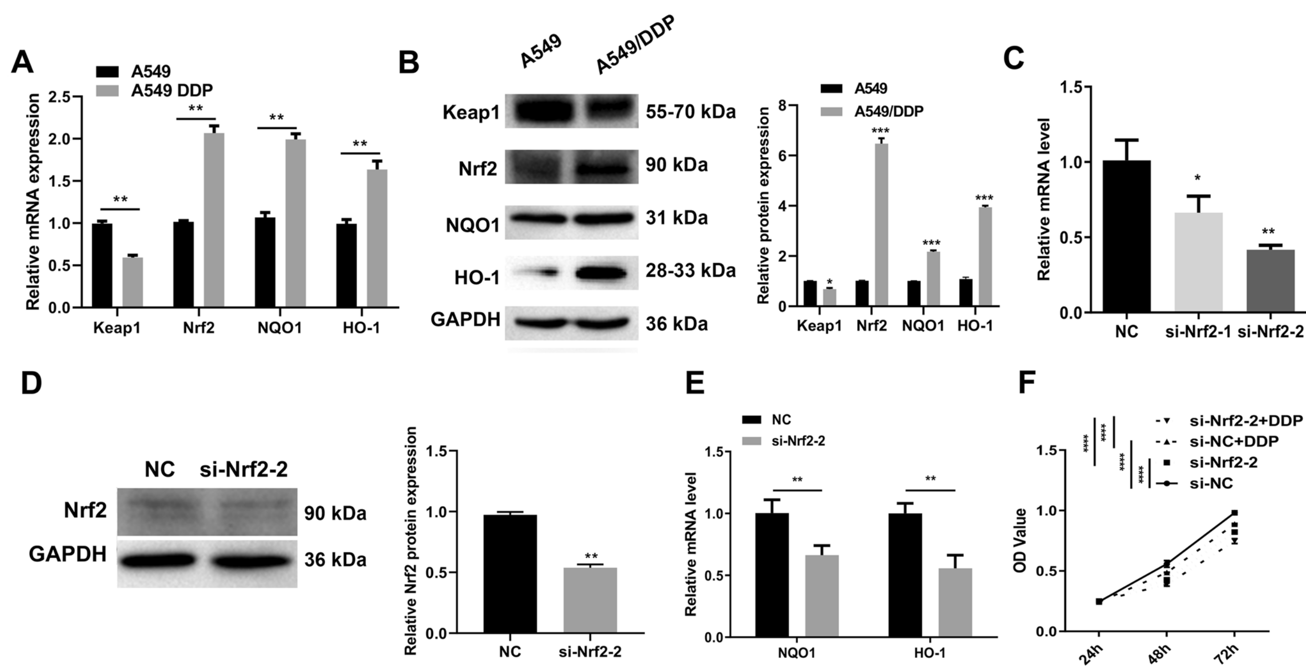

Fig. 4 The Nrf2/ARE signaling pathway was activated in A549/DDP cells. **A** Nrf2/ARE signaling pathway-related factor (Keap1, Nrf2, HO-1, and NQO1) expression in A549 and A549/DDP cells was assessed by qRT−PCR. **B** Keap1, Nrf2, HO-1, and NQO1 expression in A549 and A549/DDP cells was examined by Western blotting. The level of Nrf2 was evaluated by qRT−PCR (**C**) and Western blotting (**D**) to verify the interference efficiency of Nrf2. **E** After Nrf2 interference, NQO1 and HO-1 expression was assessed through qRT−PCR. **F** After the cells were transfected with Nrf2 interference fragments and treated with DDP, the proliferation of the A549/DDP cells was measured via the MTS assay. The quantitative data were obtained from three independent replicate experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$

that hsa_circ_0096157 silencing downregulated hsa_circ_0096157, Nrf2, and HO-1 in A549/DDP cells, which could also be significantly restored by hsa_circ_0096157 overexpression, suggesting that hsa_circ_0096157 was related to the Nrf2/ARE pathway (Fig. S2). Next, we interfered with Nrf2. Figure 4C showed that si-Nrf2-1 and si-Nrf2-2 significantly reduced Nrf2 expression, especially si-Nrf2-2. Therefore, si-Nrf2-2 was selected for subsequent interference. Western blot analysis revealed that the protein level of Nrf2 was lower in the si-Nrf2-2 group than in the NC group (Fig. 4D). Next, we detected HO-1 and NQO1 levels after Nrf2 interference. HO-1 and NQO1 expression in the A549/DDP-si-Nrf2-2 group was lower than that in the A549/DDP-si-NC-2 group (Fig. 4E). In addition, we treated A549/DDP cells with DDP while transfecting Nrf2-interfering fragments. Nrf2-mediated inhibition of A549/DDP cell proliferation was noticeably repressed. Moreover, proliferation also decreased after DDP treatment (Fig. 4F). This indicated that the DDP resistance of A549/DDP cells was affected by Nrf2 that Nrf2 expression stabilized cell proliferation, and that interference with Nrf2 led to decreased activity in A549/DDP cells. Overall, Nrf2 induced DDP resistance in A549/DDP cells.

Activation of the Nrf2/ARE signaling pathway promoted autophagy in A549/DDP cells

Next, we further examined the effect of DDP treatment and Nrf2-interfering fragment transfection on autophagy in A549/DDP cells. Nrf2 expression in the A549/DDP-si-NC+3 μg/mL DDP group was significantly greater than that in the A549/DDP-si-NC group. Nrf2 knockdown reduced Nrf2 expression compared with that in the A549/DDP-si-NC+3 μg/mL DDP group (Fig. 5A). In addition, compared with those in the A549/DDP-si-NC group, LC3 II/I and Beclin-1 expression were elevated, and the number of autophagosomes was increased in the A549/DDP-si-NC+3 μg/mL DDP group. After further interference with Nrf2, LC3 II/I and Beclin-1 expression was downregulated, and the number of autophagosomes was reduced (Fig. 5B, C).

Hsa_circ_0096157 activated the Nrf2/ARE pathway

Next, we investigated the effect of hsa_circ_0096157 on the Nrf2/ARE signaling pathway by transfecting fragments that interfered with hsa_circ_0096157 and using DDP treatment. Compared with those in the si-NC group,

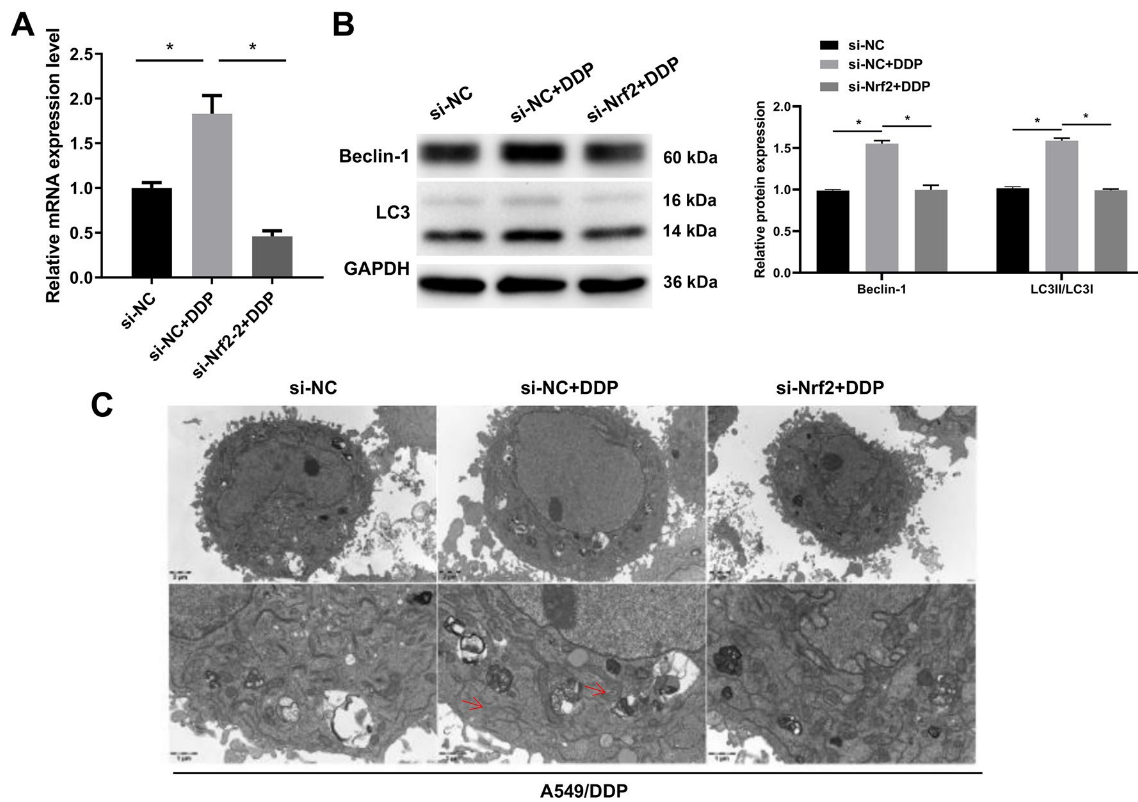

Fig. 5 Activation of the Nrf2/ARE signaling pathway promoted autophagy in A549/DDP cells. Transfection of Nrf2 fragments and treatment with DDP. **A** qRT−PCR was performed to measure Nrf2 expression. **B** Western blotting was performed to assess the expression of the autophagy-related factors LC3 and Beclin-1 in A549/DDP cells. **C** TEM assessment of autophagosomes in A549/DDP cells. The quantitative data were obtained from three independent replicate experiments. *$P < 0.05$

hsa_circ_0096157, Nrf2, and HO-1 expression increased in the si-NC+3 μg/mL DDP group; hsa_circ_0096157, Nrf2, and HO-1 levels decreased in the si-hsa_circ_0096157 group compared with those in the si-NC+3 μg/mL DDP group (Fig. 6A). In addition, compared with those in the si-NC group, Nrf2, HO-1, LC3 II/I, and Beclin-1 levels were upregulated, and p62 expression was downregulated in the si-NC+3 μg/mL DDP group; after further interference

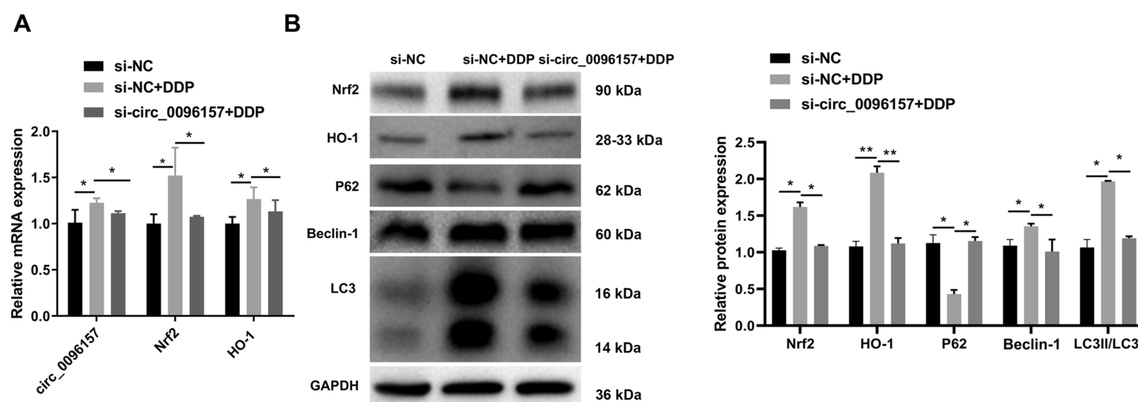

Fig. 6 Hsa_circ_0096157 activated the Nrf2/ARE signaling pathway. The cells were transfected with Nrf2 fragments and treated with DDP. **A** The expression of Hsa_circ_0096157 and Nrf2/ARE signaling pathway-related factors (Nrf2 and HO-1) was measured via qRT−PCR. **B** Western blotting was utilized to assess the expression of Nrf2/ARE signaling pathway-related factors (Nrf2 and HO-1) and autophagy-related factors (LC3, Beclin-1, and p62). The quantitative data were obtained from three independent replicate experiments. *$P < 0.05$

with hsa_circ_0096157, Nrf2, HO-1, LC3 II/I, and Beclin-1 expression was downregulated, while p62 expression was upregulated (Fig. 6B). In general, hsa_circ_0096157 activated the Nrf2/ARE pathway.

Hsa_circ_0096157 silencing downregulated Nrf2 by releasing miR-142-5p or miR-548n

To explore the possible mechanism by which hsa_circ_0096157 regulates Nrf2, we applied bioinformatics analysis to construct an hsa_circ_0096157-miRNA-Nrf2 network (Fig. 7A). Moreover, qRT−PCR data revealed that DDP downregulated miR-142-5p and miR-548n and upregulated miR-4495 in A549 cells (Fig. 7B). In addition, hsa_circ_0096157 silencing also upregulated miR-142-5p and miR-548n in A549 cells (Fig. 7C). More importantly, our data indicated that hsa_circ_0096157 silencing downregulated Nrf2, and the downregulation of Nrf2 mediated by hsa_circ_0096157 silencing could also be partially reversed by the miR-142-5p inhibitor or miR-548n inhibitor in A549 cells (Fig. 7D).

Hsa_circ_0096157 promoted autophagy by activating the Nrf2/ARE signaling pathway in A549/DDP cells

Next, we overexpressed Nrf2 in A549/DDP cells. Nrf2 expression in the A549/DDP-pcDNA3.1-Nrf2 group was significantly greater than that in the A549/DDP-pcDNA3.1 group, indicating that Nrf2 was successfully overexpressed (Fig. 8A, B). Compared with those in the si-NC+pcDNA3.1+DDP group, Nrf2 and HO-1 expression in the si-circ_0096175+pcDNA3.1+DDP group decreased. Nrf2 overexpression reversed the significant downregulation of Nrf2 and HO-1 expression caused by hsa_circ_0096157 interference (Fig. 8C). Compared with those in the si-NC+pcDNA3.1+DDP group, Nrf2, HO-1, LC3 II/I, and Beclin-1 expression in the si-circ_0096175+pcDNA3.1+DDP group was downregulated, p62 expression was upregulated, and the number of autophagosomes in A549/DDP cells was reduced. Moreover, overexpression of Nrf2 reversed the changes in the expression of these indicators and increased the number

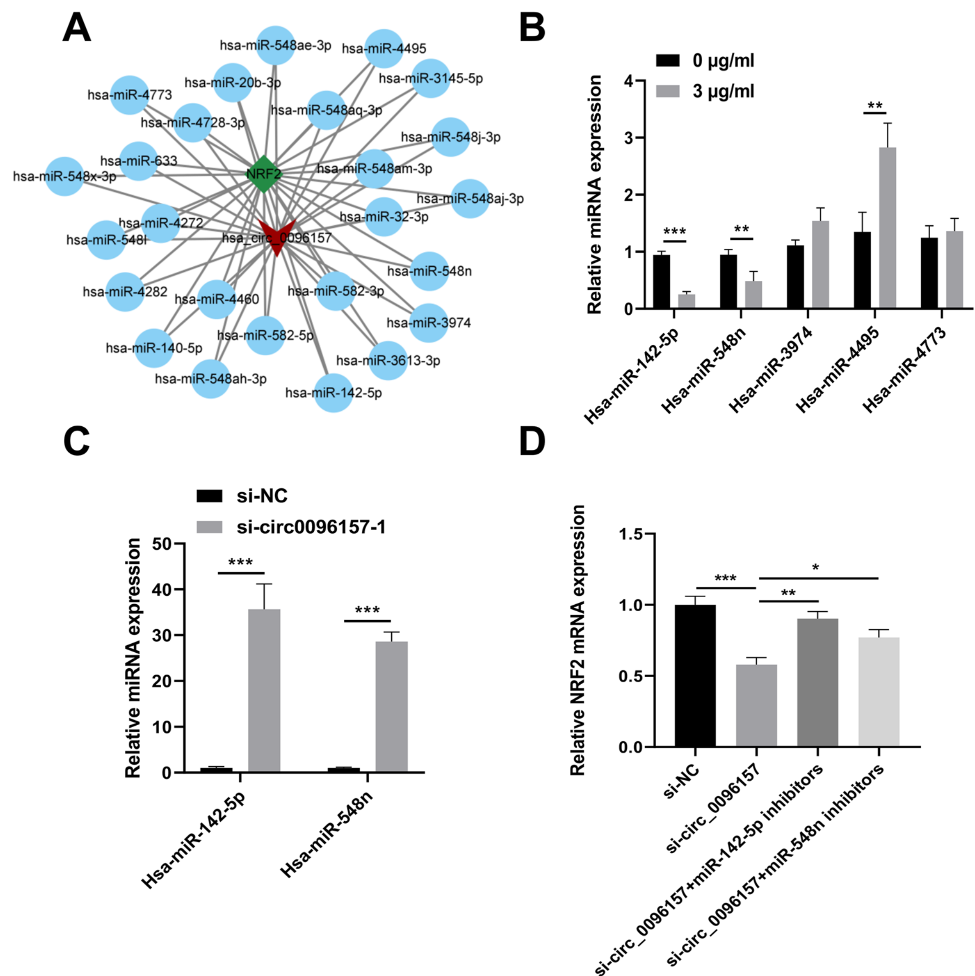

Fig. 7 Hsa_circ_0096157 silencing downregulated Nrf2 by releasing miR-142-5p or miR-548n. **A** Network diagram of hsa_circ_0096157-miRNA-Nrf2. **B** The miR-142-5p, miR-548n, miR-3974, miR-4495, and miR-4773 levels were confirmed by qRT−PCR in 3 μg/mL DDP-treated A549 cells. **C** The levels of miR-142-5p and miR-548n were tested by qRT−PCR in circ_0096157-silenced A549 cells. **D** Nrf2 expression was examined by qRT−PCR in A549 cells transfected with si-circ_0096157 and miR-142-5p inhibitor or miR-548n inhibitor. The quantitative data were obtained from three independent replicate experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$

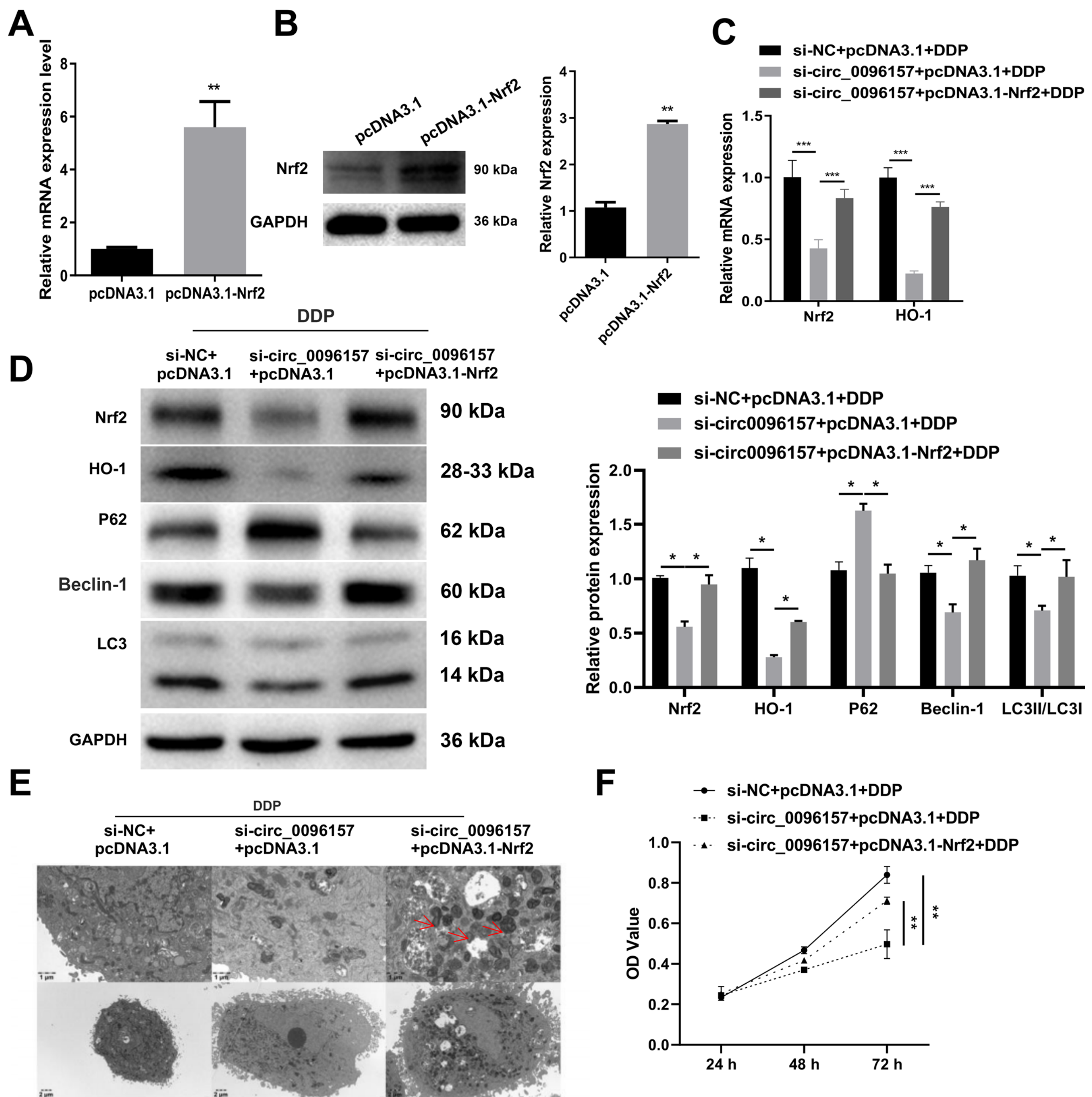

Fig. 8 Hsa_circ_0096157 promoted A549/DDP cell autophagy by activating the Nrf2/ARE pathway. **A**, **B** qRT−PCR and western blotting were used to assess the efficiency of Nrf2 overexpression. **C** qRT−PCR was utilized to measure Nrf2/ARE signaling pathway-related factor (Nrf2 and HO-1) levels in cells overexpressing hsa_circ_0096157 followed by DDP treatment. **D** Western blotting was used to measure the expression of Nrf2/ARE signaling pathway-related factors (Nrf2 and HO-1) and autophagy-related factors (LC3, Beclin-1, and p62). **E** TEM image of autophagosomes in A549/DDP cells. **F** Proliferation of A549/DDP cells was monitored by the MTS assay. The quantitative data were obtained from three independent replicate experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$

of autophagosomes in A549/DDP cells (Fig. 8D, E). In addition, after interference with hsa_circ_0096157, A549/DDP cell proliferation decreased, and DDP resistance decreased notably; in combination with overexpression of Nrf2, cell proliferation increased (Fig. 8F). Taken together, these findings indicated that hsa_circ_0096157 promoted autophagy in A549/DDP cells by activating the Nrf2/ARE pathway.

Discussion

NSCLC is a common and fatal malignancy encompassing 85% of lung tumors [19]. Treatment of tumors may fail and complicate cancer therapy because people have different symptoms and responses to specific cancer types [20]. However, chemotherapy resistance and metastasis are the main challenges in the current treatment of NSCLC [21]. Therefore, exploring the molecular pathogenesis of NSCLC could help identify targets for diagnosing and treating this disease. In recent research, we revealed that hsa_circ_0096157 activated the Nrf2/ARE signaling pathway to positively regulate autophagy and promote DDP resistance in NSCLC through in vitro validation. This is the first report of a mechanistic study of hsa_circ_0096157/Nrf2/ARE in NSCLC.

As drug therapy progresses, the emergence and development of drug resistance severely affect patient prognosis [22]. Treatment failure and disease progression in NSCLC are mainly caused by drug resistance [23]. DDP is a widely used chemotherapeutic agent for various types of human malignancies. However, the widespread use of this drug is hampered by the development of chemoresistance in some treated patients [24]. Reduction of drug accumulation in cancer cells, inactivation of the drug by reaction with glutathione and metallothionein, and faster DNA damage repair are responsible for DDP resistance [25]. In this study, we treated A549 cells with DDP and found that DDP treatment increased hsa_circ_0096157 expression and promoted autophagy and DDP resistance in NSCLC cells. This finding suggested that hsa_circ_0096157 may be related to autophagy and DDP resistance in NSCLC. Thus, we next further explored the mechanism of hsa_circ_0096157.

Previous studies have shown that knocking down hsa_circ_0096157 inhibited A549/DDP cell proliferation while increasing the apoptosis of A549/DDP cells and inhibiting DDP resistance [26]. In this study, we further illustrated that interference with hsa_circ_0096157 inhibited autophagy and DDP resistance in A549/DDP cells. Autophagic and apoptotic processes are crucial in the pathophysiology of NSCLC, where they induce protein and organelle degradation or cell death in response to cellular stress [27, 28]. Study has shown that circRNAs influence disease processes by regulating autophagy, which suggests that autophagy participates in the development of various diseases and can influence drug resistance (such as DDP resistance in tumors) [29]. Kong et al. reported that hsa_circ_0085131 was involved in DDP resistance by regulating autophagy in NSCLC cells [30]. Zhong et al. revealed that circRNA_100565 enhanced DDP resistance in NSCLC cells by regulating cell proliferation, apoptosis, and autophagy via the miR-337-3p/ADAM28 axis [31]. However, the mechanism by which hsa_circ_0096157 affects autophagy in A549/DDP cells is unclear. The addition of the autophagy activator rapamycin reversed the effect of hsa_circ_0096157 knockdown on autophagy and DDP resistance in A549/DDP cells. Therefore, we further explored the mechanism of hsa_circ_0096157, autophagy, and DDP resistance in NSCLC.

The Nrf2/ARE pathway is a protective molecular mechanism in oxidative stress [26]. Nrf2 is an essential protein that regulates the oxidative stress state of cells, coordinating the cytoprotective response to oxidative and other cellular stresses [27, 28]. Studies have shown that cancer cells develop chemoresistance when Nrf2 is activated, which inhibits drug-mediated oxidative stress and usually causes cancer cell death [32]. In NSCLC patients, dysregulated Nrf2 signaling is thought to be a common feature at the DNA and protein levels [32]. During malignant transformation, abnormal Keap1/Nrf2 activity is generally observed in NSCLC, suggesting that cell/tissue origin may influence genetic selection [33]. In this study, Nrf2 protein aboundance was also regulated by Keap1. Additionally, we showed that the Nrf2/ARE signaling pathway was activated in A549/DDP cells. Moreover, activating the Nrf2/ARE pathway promoted autophagy in A549/DDP cells. Thus, we wanted to explore whether hsa_circ_0096157 acted on the Nrf2/ARE signaling pathway to affect DDP resistance in NSCLC. We further confirmed that hsa_circ_0096157 activated the Nrf2/ARE signaling pathway. In addition, hsa_circ_0096157 promoted A549/DDP cell autophagy by activating the Nrf2/ARE signaling pathway. This is the first study on hsa_circ_0096157, the Nrf2/ARE signaling pathway, and DDP resistance in NSCLC.

Additionally, we explored the possible mechanism by which hsa_circ_0096157 regulates Nrf2. circRNAs belong to endogenous ncRNA, which can act as a competing endogenous RNA (ceRNA) to combine with microRNA (miRNA) to regulate the expression of target genes. This complementary base pairing is called the "sponge" effect. Our study screened miRNAs with targeted binding sites for hsa_circ_0096157 and Nrf2. Our data revealed that the knockdown of hsa_circ_0096157 could upregulate miR-142-5p and miR-548n in A549 cells. DDP also downregulated miR-142-5p and miR-548n. In addition, we proved that hsa_circ_0096157 silencing could downregulate Nrf2 by upregulating miR-142-5p or miR-548n. Studies have also suggested that miR-142-5p can affect the proliferation of NSCLC cells [34, 35]. Moreover, a study revealed that miR-548n was upregulated in colorectal cancer [36], contrary to our research results. Therefore, we preliminarily confirmed that hsa_circ_0096157 can regulate the expression of Nrf2, as Nrf2 may be a target of miR-142-5p and miR-548n.

However, the current study also has several limitations. For example, the role of hsa_circ_0096157 in the mTOR pathway, whether hsa_circ_0096157 can target miR-142-5p/miR-548n, whether miR-142-5p/miR-548n can target Nrf2, and the effects of hsa_circ_0096157 overexpression on cellular autophagy and DDP resistance in A549/DDP cells need to be further explored, and the impacts of hsa_circ_0096157 overexpression on Nrf2 and hsa-miR-142-5p/miR-548n also need to be further studied; these effects also need to be further investigated in animal models in the future.

Conclusion

In this study, we first confirmed that hsa_circ_0096157 silencing inhibits autophagy and DDP resistance in NSCLC cells by inhibiting the Nrf2/ARE signaling pathway. Nrf2 was regulated in two ways: Keap1 regulated the Nrf2 protein level, and hsa_circ_0096157 regulated the mRNA expression of Nrf2 maybe as a target of miR-142-5p/miR-548n. Our study provides new ideas and targets for the treatment of NSCLC.

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Author contributions All authors contributed to the study's conception and design. Cell culture and treatment, WB blot, and related analysis were performed by HL, JK, SC, HH, JL. The design of the siRNAs and TEM experiment was performed by LL. The first draft of the manuscript was written by HL, and all authors commented on previous versions. All authors read and approved the final manuscript.

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

Declarations

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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