Original Article

p32/OPA1 axis-mediated mitochondrial dynamics contributes to cisplatin resistance in non-small cell lung cancer

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Abstract

Cisplatin resistance is a major obstacle in the treatment of non-small cell lung cancer (NSCLC). p32 and OPA1 are the key regulators of mitochondrial morphology and function. This study aims to investigate the role of the p32/OPA1 axis in cisplatin resistance in NSCLC and its underlying mechanism. The levels of p32 protein and mitochondrial fusion protein OPA1 are higher in cisplatin-resistant A549/DDP cells than in cisplatin-sensitive A549 cells, which facilitates mitochondrial fusion in A549/DDP cells. In addition, the expression of p32 and OPA1 protein is also upregulated in A549 cells during the development of cisplatin resistance. Moreover, *p32* knockdown effectively downregulates the expression of OPA1, stimulates mitochondrial fission, decreases ATP generation and sensitizes A549/DDP cells to cisplatin-induced apoptosis. Furthermore, metformin significantly downregulates the expressions of p32 and OPA1 and induces mitochondrial fission and a decrease in ATP level in A549/DDP cells. The co-administration of metformin and cisplatin shows a significantly greater decrease in A549/DDP cell viability than cisplatin treatment alone. Moreover, D-erythro-Sphingosine, a potent p32 kinase activator, counteracts the metformin-induced downregulation of OPA1 and mitochondrial fission in A549/DDP cells. Taken together, these findings indicate that p32/OPA1 axis-mediated mitochondrial dynamics contributes to the acquired cisplatin resistance in NSCLC and that metformin resensitizes NSCLC to cisplatin, suggesting that targeting p32 and mitochondrial dynamics is an effective strategy for the prevention of cisplatin resistance.

Key words p32, OPA1, mitochondrial dynamics, cisplatin resistance, metformin, non-small cell lung cancer

Introduction

Lung cancer is one of the most common tumors in the world, with a poor prognosis and a high mortality [1], and accounts for approximately a quarter of all cancer fatalities with a less than 20% 5-year survival rate [2,3]. The primary subtypes of lung cancer include non-small cell lung cancer (NSCLC, approximately 85%) and small cell lung cancer (approximately 15%) [4]. Current therapeutic strategies for NSCLC include surgery, chemotherapy, radiotherapy and immunotherapy. However, since NSCLCs are often diagnosed at a late stage, patients usually lose the chance for radical surgery, and chemotherapy becomes the main treatment. Cis-diamminedichloroplatinum (cisplatin, DDP) is the most effective and first-line chemotherapeutic agent for patients with NSCLC [5]. However, long-term use of cisplatin eventually leads to cisplatin resistance in most cases, resulting in NSCLC recurrence, metastasis and poor prognosis [6]. Emerging evidence indicates that mito-

chondria are the target organelles of cisplatin and play an important role in cisplatin-induced cytotoxicity in cancer cells [7,8]. Therefore, it is urgent to investigate the role of mitochondria in the development of cisplatin resistance in NSCLC.

Mitochondria are highly dynamic organelles with coordinated cycles of division, elongation and fusion, known as "mitochondrial dynamics" [9]. Mitochondrial dynamics, regulated by fission and fusion, depend on dynamin-related GTPase protein 1 (Drp1), a key regulator of mitochondrial fission, and optic atrophy 1 (OPA1) and mitofusin 1/2 (Mfn1/2), which mediate the fusion of inner and outer mitochondrial membranes, respectively [9]. Mitochondrial dynamics have been implicated in resistance or sensitivity to chemotherapies. Mitochondrial fusion facilitates the formation of a tubular network of mitochondria, which counteracts metabolic insults, maintains cell integrity, and prevents mitosis and apoptosis [10]. Instead, mitochondrial fission produces a large number of small and broken mitochondria, which are implicated in the stress response and apoptosis [11]. It has been reported that mitochondrial fusion may be associated with cisplatin resistance in mouse leukemia L1210 cells [12]. Chemo-resistant cells have a higher proportion of reticular mitochondria and higher levels of mitochondrial fusion in gynecological cancers [13]. Overexpression of OPA1 increases cisplatin resistance in lung adenocarcinoma [14], whereas DRP1-dependent mitochondrial division improves cisplatin sensitivity in lung cancer [15]. Thus, mitochondrial fusion is linked to chemotherapy resistance in several cancers, with recent studies suggesting its importance in acquiring cisplatin resistance in NSCLC.

p32/gC1qR/C1QBP/HABP1, a chaperone-like protein, is mainly located in the mitochondrial matrix and regulates mitochondrial structure and functions, which is essential for the maintenance of mitochondrial oxidative phosphorylation [16]. In neurons, p32 is involved in mitochondrial dynamics and morphology through Parkin [17]. In mouse embryonic fibroblasts, loss of p32 leads to the disruption of mitochondrial membrane potential, activation of OMA1, cleavage of OPA1, and mitochondrial swelling or rupture [18]. Notably, overexpression of p32 has been reported in a variety of tumors and is related to tumor stage [19]. However, the relationship between p32 and cisplatin resistance and its underlying mechanisms remain to be elucidated.

Here, we found that mitochondrial fusion was increased in cisplatin-resistant A549/DDP cells. The expression of p32 and OPA1 was upregulated with the progression of cisplatin resistance in A549 cells. Knockdown of *p32* downregulated OPA1 expression, induced mitochondrial fission, decreased ATP production and sensitized A549/DDP cells to cisplatin. Metformin treatment downregulated the expressions of p32 and OPA1, induced mitochondrial fission and resensitized A549/DDP cells to cisplatin. Intriguingly, activation of p32 by D-erythro-Sphingosine effectively prevented metformin-induced downregulation of OPA1 and mitochondrial fission in A549/DDP cells. Therefore, we propose metformin as a potential agent to sensitize NSCLC to cisplatin by suppressing p32/OPA1 axis-mediated mitochondrial dynamics.

Materials and Methods
Cell culture
The human NSCLC cell lines A549 and H460 were obtained from the National Collection of Authenticated Cell Cultures of the Chinese Academy of Sciences (Shanghai, China). Cisplatin-resistant A549/DDP cells were kindly provided by Professor Yong Li from the First Affiliated Hospital of Nanchang University, which were purchased from Procell Life Science ,Technology Co., Ltd (Wuhan, China). A549 and H460 cells were cultured in RPMI 1640 (BI, Beit HaEmek, Israel) supplemented with 10% fetal bovine serum (FBS; BI, Beit HaEmek, Israel) and 1% penicillin/streptomycin (P/S; Solarbio, Beijing, China) at 37°C in a humidified atmosphere with 5% CO$_2$. A549/DDP cells were cultured in the same medium containing 2 μg/mL cisplatin (#HY-17394; MCE, Shanghai, China) to maintain the cisplatin-resistant phenotype. Cells were cultured in complete medium without cisplatin for 3 days before any experiment was performed.

Cell viability assay
A CCK-8 assay kit (ApexBio, Houston, USA) was used to assess the cytotoxicity of cisplatin in A549 and A549/DDP cells. Briefly, cells were seeded in 96-well plates and treated with the indicated drugs. Next, 10 μL of CCK-8 buffer was added to each well and incubated at 37°C for 2 h. The absorbance in each well was then examined at 450 nm via a microplate reader (EnSpire; Perkin Elmer, Shanghai, China). All assays were performed at least three times.

Western blot analysis
Cells were treated with the Metformin (HY-B0627; MCE, Shanghai, China)and D-erythro-Sphingosine (HY-101047; MCE) Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated at 4°C with RIPA buffer supplemented with a proteinase inhibitor cocktail (#P6730; Solarbio, Beijing, China) for 30 min. Subsequently, the samples were centrifuged at 4°C and 14,000 *g* for 15 min, and the supernatant was collected for 10% SDS-PAGE. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, USA). After being blocked with 5% skim milk, PVDF membranes were incubated with the following primary antibodies overnight at 4°C: p32 (#6502, 1:1000; Cell Signaling Technology, Danvers, USA), OPA1 (#80471, 1:1000; Cell Signaling Technology), Drp1 (#8570, 1:1000; Cell Signaling Technology), and β-actin (#AC004, 1:100,000; Abconal). Next, PVDF membranes were incubated with HRP-conjugated secondary antibody (#B900210, 1:5000; Proteintech). Protein bands were finally detected using an enhanced chemiluminescence (ECL) detection kit (#KF8001; Affinity). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, USA). β-Actin was used as the loading control.

RNA interference
Small interfering RNAs were used for the knockdown of *p32* and *OPA1* in A549/DDP cells. siRNAs targeting p32 were purchased from Ribo Life Science (Suzhou, China). siRNAs targeting OPA1 were purchased from Biomics (Nantong, China). Sequences of siRNA targeted p32 and OPA1 were as follows: p32-si-1 sense 5′-GGACAACACUUUUGCAGAUdTdT-3′, antisense 5′-AUCUGCAAAAGUGUUGUCCdTdT-3′; p32-si-2 sense 5′-GAGUCUGAAUGGAAGGAUAdTdT-3′, antisense 5′-UAUCCUUCCAUUCAGACUCdTdT-3′; p32-si-3 sense: 5′-GUUGGACUGUCAUUAUCCAdTdT-3′, antisense 5′-UGGAUAAUGACAGUCCAACdTdT-3′; p32 NC (siN0000001-1-5); OPA1-si-1 sense 5′-CCGGACCUUAGUGAAUAUAdTdT-3′, antisense 5′-UAUAUUCACUAAGGUCCGGdTdT-3′; OPA1-si-2 sense 5′-GUGGCCCUAUUUAAAGAUAdTdT-3′, antisense 5′-UAUCUUUAAAUAGGGCCACdTdT-3′; OPA1-si-3 sense 5′-GUGGGAAAGAGUAUCAACUdTdT-3′, antisense 5′-AGUUGAUA

CUCUUUCCCACdTdT-3′; OPA1-si-NC sense 5′-UUCUCCGAAC GUGUCACGUdTdT-3′, antisense 5′-ACGUGACACGUUCGGA GAAdTdT-3′. siRNAs were transfected into A549/DDP cells using a riboFECT CP Transfection kit (#C10511-05; Ribo Life Science) according to the manufacturer's instructions.

Annexin V-FITC/PI apoptosis assay

A FITC Annexin V Apoptosis Detection kit (#556547; BD Biosciences, Beijing, China) was used to detect apoptosis according to the manufacturer's instructions. After the indicated treatments, cells were harvested and resuspended in 300 μL of binding buffer with 5 μL of propidium iodide (PI) and 5 μL of Annexin V-FITC, and incubated at room temperature for 15 min in the dark. Then, the samples were subjected to the apoptosis assay by flow cytometry, and the data were analyzed with FlowJo software (BD Biosciences).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was detected using tetra-methylrhodamine ethyl ester (TMRE; Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Briefly, cells were incubated with 100 nM TMRE for 20 min at 37°C and washed with PBS twice. Fluorescence images were acquired with an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Mitochondrial imaging and analysis

To label mitochondria, cells were transfected with pDsRed2-Mito (Clontech Laboratories) according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen). After 7 h of incubation, the medium containing Lipofectamine was replaced by complete culture medium supplemented with 10% FBS. To examine the role of p32 in mitochondrial dynamics, cells were transfected with p32 siRNA. Mitochondrial morphology was examined under a FV3000 confocal microscope (Olympus), and mitochondrial length was measured using ImageJ software. For each group, approximately 270 mitochondria from 5 to 8 different random fields were measured, and the average length of mitochondria was calculated.

Intracellular ATP level assay

A luciferase-based kit (S0026B; Beyotime Biotechnology, Shanghai, China) was used to measure ATP level according to the manufacturer's instructions. After centrifugation at 4°C and 12,000 g for 5 min, the supernatant was carefully aspirated and placed on ice. Meanwhile, an aliquot (100 μL) of ATP detection working solution was added to each well of a black tissue culture-treated 96-well plate and incubated for 3 min at room temperature. Then, two replicates of 20 μL samples of the cell lysate from each group were added to the wells, and luminescence values were measured. In addition, serial dilutions of a standard ATP solution were measured to generate a standard curve, and the ATP levels in cells were calculated by comparison with the ATP standard curve.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 7, and the data are expressed as the mean ± SD. Statistical comparisons were conducted by one-way analysis of variance (ANOVA, for comparisons of three or more groups) followed by Tukey's *post hoc* test or Student's *t* test (for comparisons between two groups). $P < 0.05$ was considered statistically significant.

Results

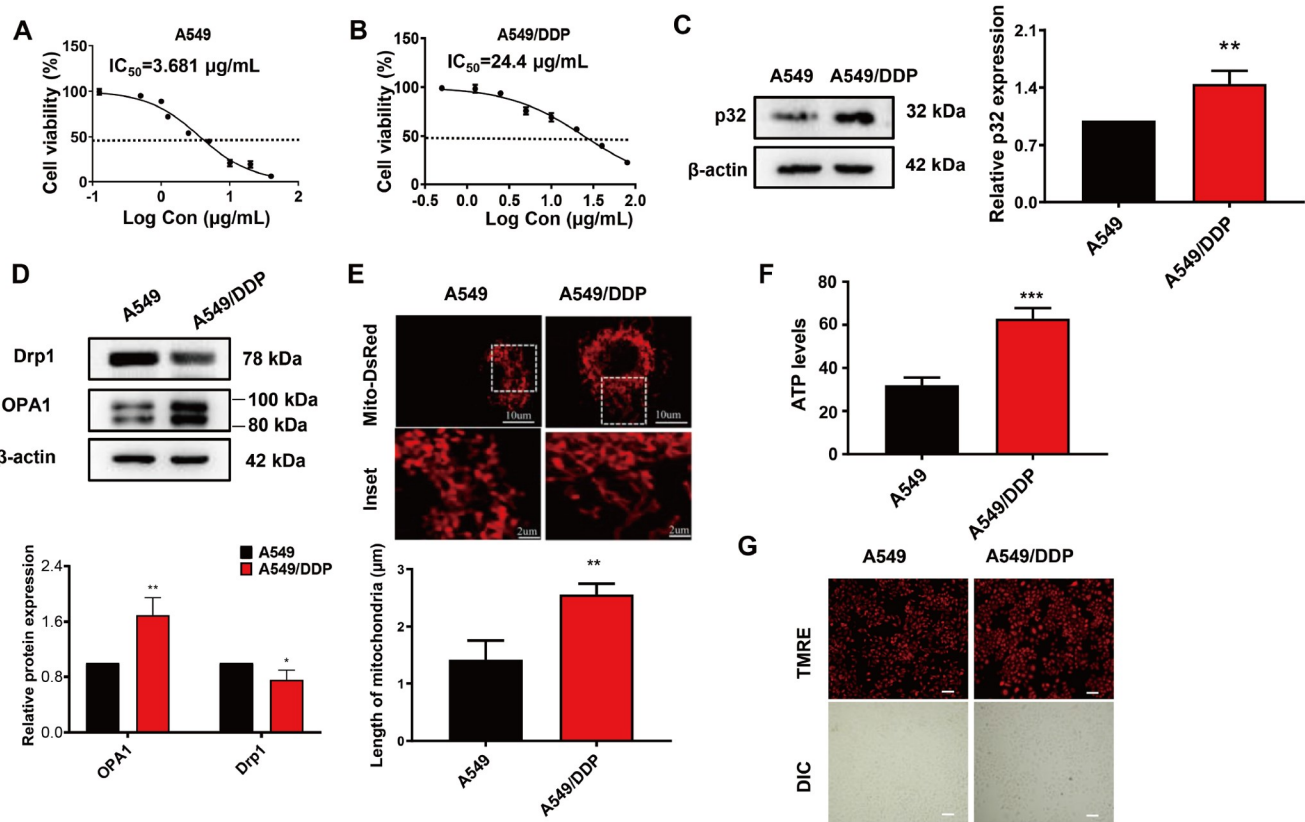### p32 expression and mitochondrial dynamics are related to cisplatin resistance in NSCLC

The IC$_{50}$ values of cisplatin for A549 and A549/DDP cells were determined by CCK8 assay. The dose-response curves of A549 and A549/DDP cell viability were generated with cisplatin treatments at different concentrations (Figure 1A,B), and the IC$_{50}$ values were determined to be 3.68 and 24.4 μg/mL in A549 and A549/DDP cells, respectively. Compared to that for A549 cells, the IC50 value of cisplatin for A549/DDP cells was increased by almost 7-folds. To investigate the role of p32 and mitochondrial dynamics in cisplatin resistance in NSCLC, p32 expression was examined in A549 and A549/DDP cells, and the results showed a higher level of p32 in A549/DDP cells than in A549 cells (Figure 1C). Furthermore, the expression of OPA1 was also upregulated, while Drp1 was down-regulated in A549/DDP cells (Figure 1D). To investigate the changes in mitochondrial morphology, pDsRed2-Mito was transfected into A549 and A549/DDP cells for mitochondrial imaging. As shown in Figure 1E, mitochondria in A549/DDP cells appeared as tubular, thread-like networks, and the average length of mitochondrial fragments was significantly longer than that in A549 cells. In addition, the ATP level in A549/DDP cells was higher than that in A549 cells (Figure 1F). However, there was no significant difference between A549 and A549/DDP cells in mitochondrial membrane potential, as indicated by TMRE staining (Figure 1G). Taken together, these results suggest that p32 and mitochondrial dynamics may be related to cisplatin resistance in NSCLC.

p32 and mitochondrial fusion protein OPA1 are upregulated in NSCLC cells during the development of cisplatin resistance

To simulate the process of cisplatin resistance, A549 cells were treated with 2.5 μg/mL cisplatin, and the expressions of p32, OPA1 and Drp1 were examined at days 0, 2, 4 and 6 after cisplatin treatment. As shown in Figure 2A,C, the expressions of p32 and OPA1 were upregulated on Day 6 after cisplatin treatment. In contrast, the expression of Drp1 was not significantly changed following cisplatin treatment, suggesting that the mitochondrial fission process was not altered (Figure 2B). Furthermore, similar results were obtained in H460 cells, as cisplatin treatment also increased the expressions of p32 and OPA1 in H460 cells on Day 6 (Supplementary Figure S1). These results suggest that p32 and OPA1 may be involved in acquired cisplatin resistance in NSCLC cells.

Knockdown of *p32* resensitizes A549/DDP cells to cisplatin-induced cytotoxicity

To downregulate the expression of p32, siRNAs (siRNA-1, siRNA-2, and siRNA-3) targeting p32 were transfected into A549/DDP cells. As shown in Figure 3A, siRNAs significantly downregulated p32 expression. siRNA-2 was selected for the subsequent experiments due to its optimal knockdown efficiency. To examine the role of p32 in cisplatin resistance, A549/DDP cells were transfected with p32 siRNA or treated with cisplatin, and cell viability was examined by CCK-8 assay. DDP at 10 μg/mL significantly decreased A549/DDP cell viability to approximately 70% of the control. Although *p32* knockdown alone had no significant effect on cell viability, DDP in combination with *p32* knockdown showed greater inhibitory efficiency on cell viability than DDP treatment alone (Figure 3B).

Figure 1. p32 and mitochondrial dynamics are related to cisplatin resistance in A549 cells (A) The dose-response curve of A549 cells to cisplatin. (B) The dose-response curve of A549/DDP cells to cisplatin. The cells were treated with cisplatin for 24 h, and cell viability was measured by CCK-8 assay. (C,D) Expressions of p32, Drp1 and OPA1 in A549 and A549/DDP cells. Whole cell lysates were collected for western blot analysis. β-Actin was used as a loading control. *$P < 0.05$, **$P < 0.01$ vs A549 cells. (E) Mitochondrial morphology in A549 and A549/DDP cells. The cells were transfected with pDsRed2-Mito, and the fluorescence signal of pDsRed2-Mito was used to label mitochondria and delineate mitochondrial morphology. The length of mitochondria in each group was measured in μm. Data are presented as the mean ± SD of three independent experiments. **$P < 0.01$. (F) ATP level was determined by luminescence assay in A549 and A549/DDP cells. ***$P < 0.001$. (G) Mitochondrial membrane potential was detected using TMRE reagent. Fluorescence images were acquired with an inverted microscope. Scale bar: 100 μm.
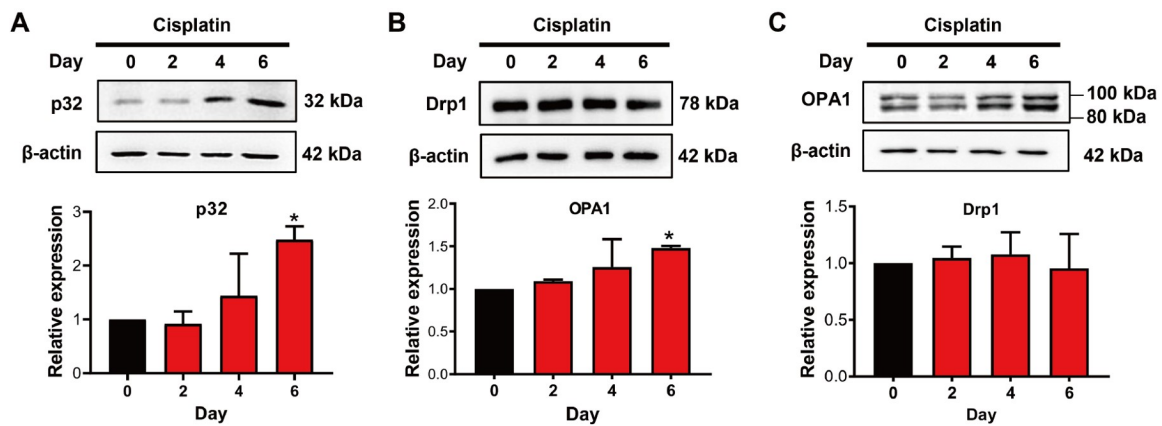

Figure 2. Cisplatin stress upregulates the expressions of p32 and OPA1 in A549 cells After exposure to 2.5 μg/mL cisplatin (< IC$_{50}$) for 0, 2, 4 and 6 days, A549 cells were harvested. Whole cell lysates were collected for western blot analysis to detect the expressions of p32 (A), Drp1 (B) and OPA1 (C). β-Actin was used as a loading control. *$P < 0.05$ vs Day 0.

Similarly, DDP treatment, but not knockdown of *p32*, significantly increased cell apoptosis in A549/DDP cells, and DDP in combination with *p32* knockdown induced significantly more cell apoptosis than DDP treatment alone (Figure 3C). These findings indicate that knockdown of *p32* sensitizes NSCLC cells to cisplatin.

p32/OPA1 axis-mediated mitochondrial fusion in cisplatin-resistant NSCLC cells

To further investigate the underlying mechanism of p32-mediated mitochondrial dynamics, *p32* was knocked down in A549/DDP cells by RNA interference. Western blot analysis results showed that
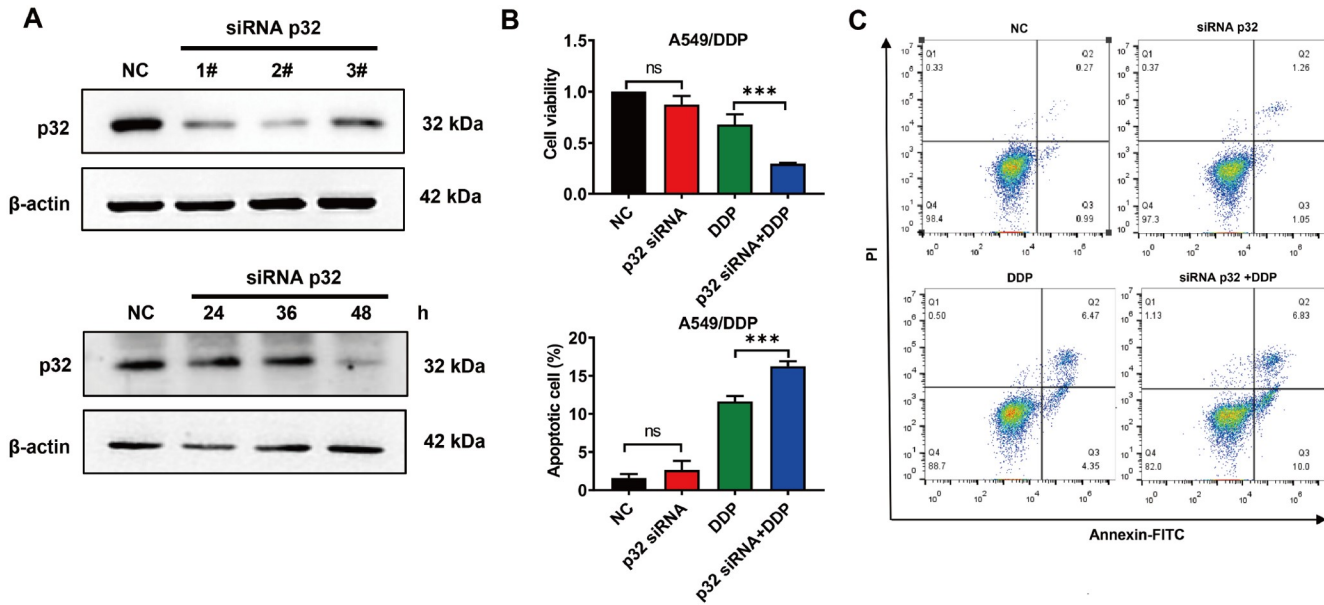
Figure 3. Knockdown of *p32* sensitizes A549/DDP cells to cisplatin-induced cytotoxicity (A) *p32* knockdown efficiency of siRNAs in A549/DDP cells. Cells were transfected with p32 siRNAs or scramble RNA (NC). Whole cell lysates were collected for western blot analysis to detect the expression of p32. β-Actin was used as a loading control. The top panels show the *p32* knockdown efficiency of different siRNAs, and the bottom panels show the *p32* knockdown efficiency of siRNA #2 at different time points. (B) Effect of p32 RNAi on the cisplatin-induced decrease in cell viability in A549/DDP cells. DDP (10 μg/mL) was used to treat A549 cells without transfection or transfected with p32 siRNA for 24 h. Cell viability was determined by CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. ns: no significance, ***$P < 0.001$. (C) Effect of p32 siRNA on cisplatin-induced apoptosis in A549/DDP cells. DDP (10 μg/mL) was used to treat A59 cells without transfection or transfected with p32 siRNA for 24 h. Apoptosis was detected by flow cytometry using Annexin V-FITC/PI double staining. The percentage of apoptosis (Q2 and Q3) in each group was calculated. Data are presented as the mean ± SD of three independent experiments.

OPA1 expression was significantly decreased following *p32* knockdown in A549/DDP cells, while Drp1 expression was not changed (Figure 4A). Confocal imaging showed that *p32* knockdown significantly reduced the average length of mitochondria and induced the formation of fragmented, punctate dot-like mitochondria. OPA1 overexpression restored the length of mitochondria and resulted in tubular and thread-like mitochondria (Figure 4B), indicating that mitochondrial fusion might be mediated by the p32/OPA1 axis. Consistent with mitochondrial dynamics alterations, knockdown of *p32* in A549/DDP cells also disrupted mitochondrial membrane potential and markedly reduced ATP production, both of which were restored by OPA1 overexpression (Figure 4C,D). Furthermore, *OPA1* knockdown sensitized A549/DDP cells to cisplatin without affecting p32 expression, indicating that OPA1 acts downstream of p32 (Figure 4E and Supplementary Figure S2A,B). All these results highlight the important role of p32/OPA1 axis-mediated mitochondrial fusion in the development of cisplatin resistance in NSCLC.

Metformin resensitizes A549/DPP cells to cisplatin via the p32/OPA1 axis

It was reported that metformin had anticancer and chemosensitization effects in NSCLC [20]. Here, we explored whether metformin could sensitize NSCLC cells to cisplatin via the p32/OPA1 axis. First, CCK-8 assay was performed to examine the effect of metformin on the viability of A549/DDP cells. As shown in Figure 5A, metformin decreased the cell viability of A549/DDP cells in a dose-dependent manner. Next, the effects of metformin on the expressions of p32 and OPA1 were examined by western blot analysis. p32 and OPA1 expressions were downregulated following

metformin treatment (Figure 5B,C). Metformin also decreased intracellular ATP level and disrupted mitochondrial membrane potential in A549/DDP cells (Figure 5D,E). Mitochondria were shown in confocal microscopy imaging as punctate or dot-like structures, with a notable decrease in average mitochondrial length after metformin treatment (Figure 5F). Consistent with alterations in mitochondrial morphology and ATP generation, treatment with metformin in combination with DDP induced a significantly greater decrease in the cell viability of A549/DDP cells than treatment with metformin or DDP alone (Figure 5G). To further investigate the role of the p32/OPA1 axis in metformin-induced chemosensitization, D-erythro-Sphingosine (Sph), an agonist of p32, was used to significantly upregulate the expression of OPA1 in A549/DDP cells (Figure 6A). Notably, D-erythro-Sphingosine also alleviated metformin-induced downregulation of OPA1 and mitochondrial fission (Figure 6B,C). These results suggest that metformin sensitizes NSCLC cells to cisplatin by suppressing p32/OPA1 axis-mediated mitochondrial fusion.

Discussion

Lung cancer is the leading cause of cancer death. With cisplatin as one of the first-line chemotherapeutic agents for NSCLC, its resistance is the main reason for NSCLC recurrence. Therefore, searching for new molecular targets to prevent cisplatin resistance is crucial to improve the prognosis of NSCLC patients. It is well established that mitochondrial dynamics are involved in acquired chemotherapy resistance and that p32 is a key regulator of mitochondrial function and morphology [16,17]. Nevertheless, the role of p32 in mitochondrial dynamics-mediated cisplatin resistance remains unknown.
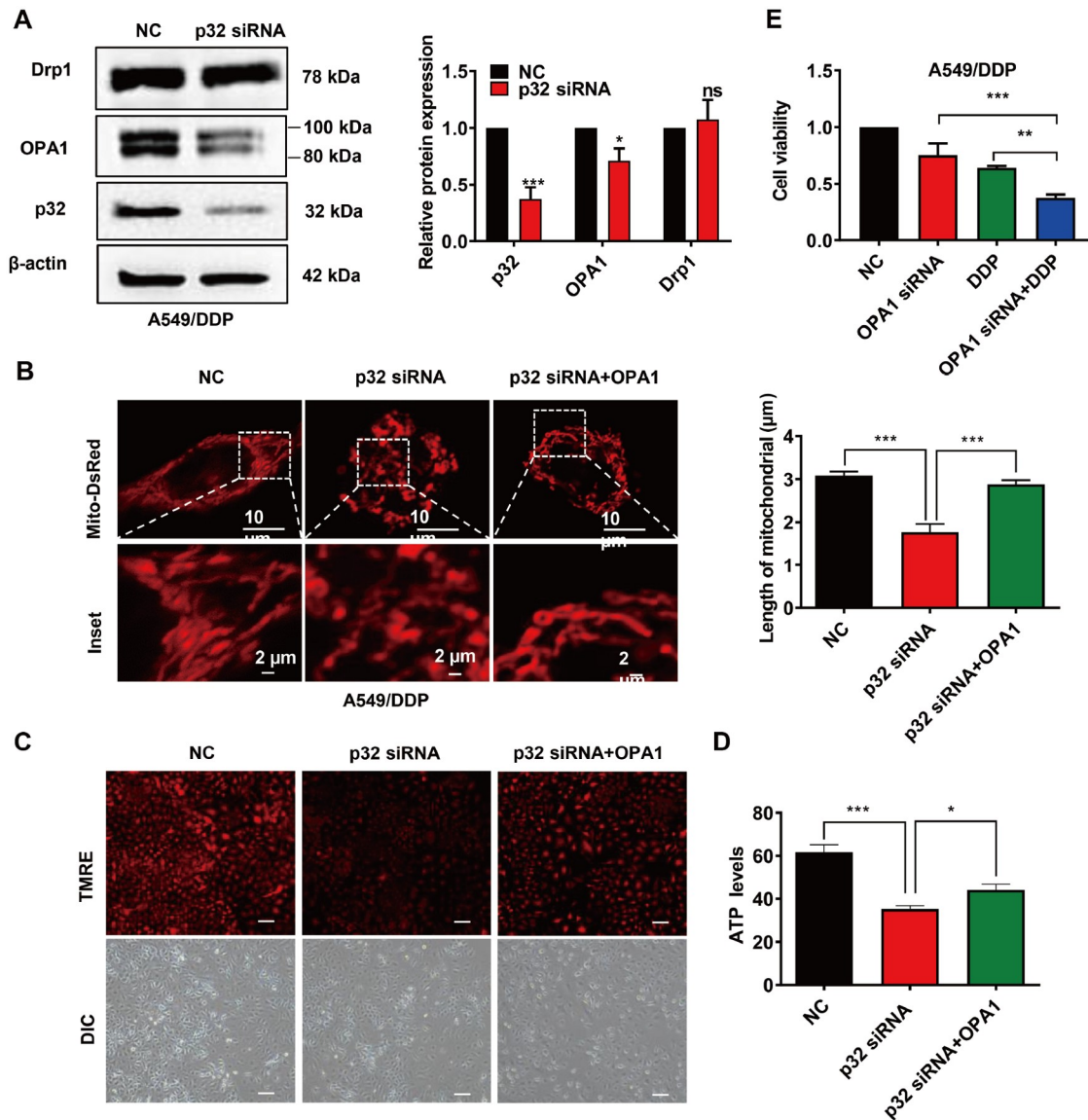
Figure 4. The p32/OPA1 axis promotes mitochondrial fusion in cisplatin-resistant A549/DDP cells (A) Effect of p32 on the expression of mitochondrial dynamics-related proteins. A549/DDP cells were transfected with p32 siRNAs or scramble RNA (NC). Whole cell lysates were collected for western blot analysis to detect the expressions of p32, OPA1 and Drp1. β-Actin was used as a loading control. *$P < 0.05$, ***$P < 0.001$ vs NC. Ns: no significance. (B) Mitochondrial morphology in A549/DDP cells transfected with p32 siRNA or both p32 siRNA and OPA1 plasmid. The cells were transfected with pDsRed2-Mito to label mitochondria, and the fluorescence signal of pDsRed2-Mito was used to label mitochondria and delineate mitochondrial morphology. The length of mitochondria in each group was measured in μm. Data are presented as the mean ± SD of three independent experiments. ***$P < 0.001$. (C) Mitochondrial potential in A549/DDP cells transfected with p32 siRNA or p32 siRNA and OPA1 plasmid. Mitochondrial membrane potential was detected using TMRE reagent. Fluorescence images were acquired with an inverted microscope. Scale bar: 100 μm. (D) ATP level in A549/DDP cells transfected with p32 siRNA or p32 siRNA and OPA1 plasmid. Cells were transfected with p32 siRNA or OPA1 plasmid for 48 h, and ATP levels were determined by a luminescence assay. *$P < 0.05$, ***$P < 0.001$. Data are presented as the mean ± SD of three independent experiments. (E) Effect of OPA1 siRNA on the cisplatin-induced decrease in cell viability in A549/DDP cells. DDP (10 μg/mL) was used to treat A59 cells without transfection or transfected with OPA1 siRNA for 24 h. Cell viability was determined by CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. **$P < 0.01$, ***$P < 0.001$.

p32 expression is elevated in most cancer types and correlates with poor prognosis [21]. For example, it has been reported that p32 is highly expressed in prostate tumors and critical for cell proliferation in prostate cancer [22], and p32 promotes cell proliferation by upregulating CKS1B transcription and inhibiting C1q in myeloma [23]. In addition, overexpression of p32 has been shown to promote the malignant phenotype of colorectal cancer cells [24]. However, whether high expression of p32 is associated

with chemotherapy resistance remains unclear. In the present study, we found that p32 expression was higher in cisplatin-resistant A549/DDP cells than in cisplatin-sensitive A549 cells. The expression of p32 was also upregulated in A549 cells under cisplatin stress, indicating that p32 is related to the acquired cisplatin resistance. Moreover, it has been reported that cisplatin can induce apoptosis by targeting mitochondria [25]. In the current study, we found that knockdown of *p32* significantly augmented cisplatin-
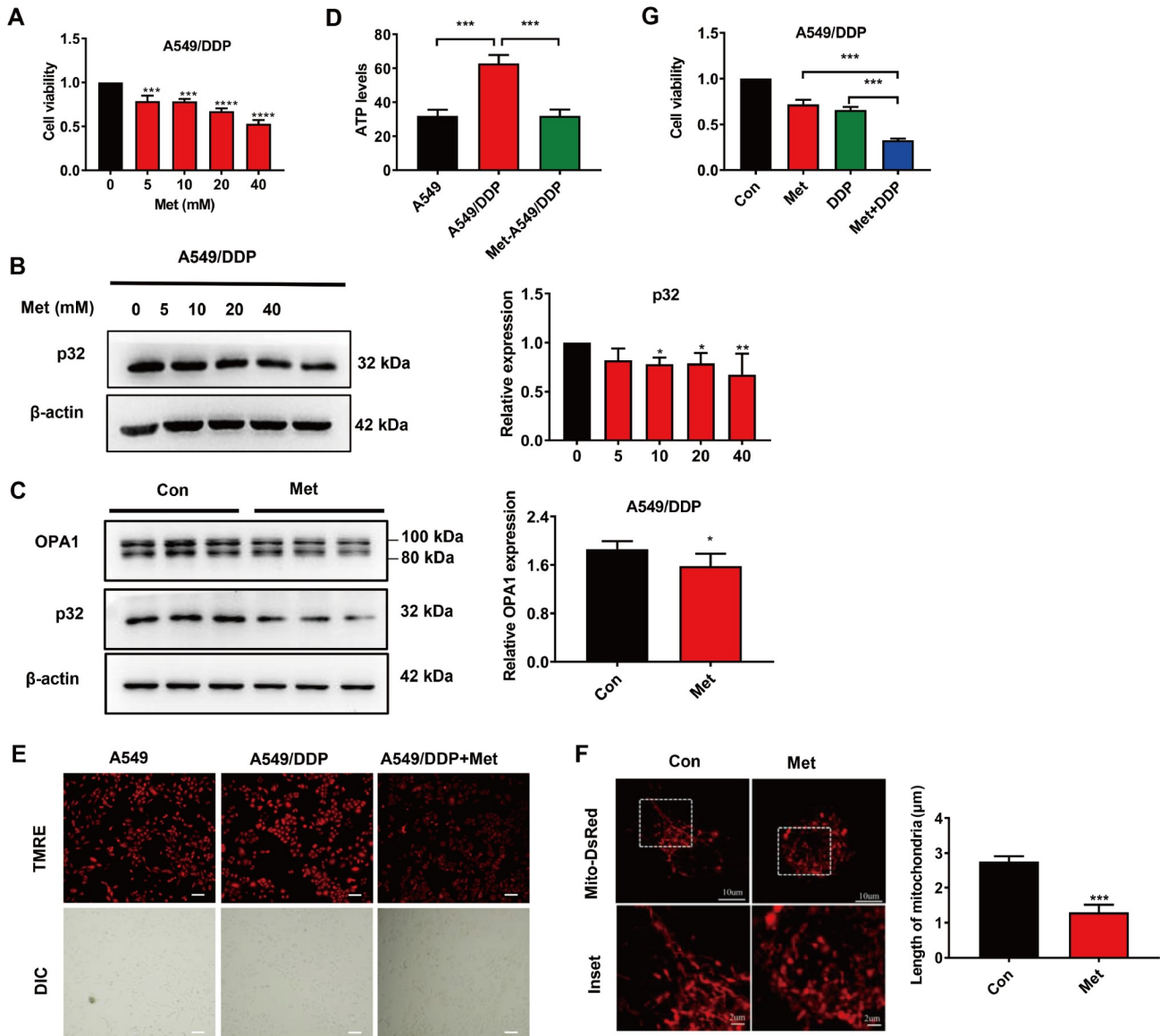
Figure 5. Metformin sensitizes A549/DPP cells to cisplatin via the p32/OPA1 axis (A) Effect of metformin on the viability of A549/DDP cells. Cells were treated with 0, 5, 10, 20 and 40 mM metformin for 24 h, and cell viability was determined by CCK-8 assay. ***$P < 0.001$, ****$P < 0.0001$ vs 0 mM Met. (B,C) Effect of metformin on the expressions of p32 and OPA1. After A549/DDP cells were treated with metformin for 24 h, whole cell lysates were collected for western blot analysis to detect the expressions of p32 (B) and OPA1 (C). β-Actin was used as a loading control. *$P < 0.05$, **$P < 0.01$. (D) Effect of metformin on intracellular ATP level. After A549 and A549/DDP cells were treated with or without 10 mM metformin for 24 h, ATP levels were determined by a luminescence assay. Data are presented as the mean ± SD of three independent experiments. ***$P < 0.001$. (E) Effect of metformin on mitochondrial membrane potential. After A549 and A549/DDP cells were treated with or without 10 mM metformin for 24 h, mitochondrial membrane potential was detected using TMRE reagent. Fluorescence images were acquired with an inverted microscope. Scale bar: 100 μm. (F) Effect of metformin on mitochondrial morphology in A549/DDP cells. Cells were transfected with pDsRed2-Mito and treated with or without 10 mM metformin for 24 h. The fluorescence signal of pDsRed2-Mito was used to label mitochondria and delineate mitochondrial morphology. The length of mitochondria in each group was measured in μm. Data are presented as the mean ± SD of three independent experiments. ***$P < 0.001$ vs Control. (G) Effect of metformin on the cisplatin-induced decrease in viability of A549/DDP cells. After cells were treated with 10 mM metformin alone or together with 10 μg/mL cisplatin, cell viability was determined by CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. ***$P < 0.001$.

induced cytotoxicity in A549/DDP cells, manifested by enhanced apoptosis and decreased cell viability, although knockdown of *p32* alone had no effect on cell viability or apoptosis. These findings suggest that p32 is a potential molecular target for preventing cisplatin resistance in NSCLC.

Mitochondria, on the other hand, are the target organelles of cisplatin, and the mitochondrial apoptosis pathway plays a key role in the chemotherapeutic effects of cisplatin in cancer treatments [7,8]. Mitochondria are also highly dynamic, and their morphology is well-balanced by fusion and fission [10]. In rat neuroblastoma B50 cells, resistance to cisplatin was found to be mediated by OPA1-mediated mitochondrial fusion [26], while inhibition of Mfn2 was shown to sensitize human neuroblastoma cells to cisplatin [27]. Our previous study revealed a connection between mitochondrial
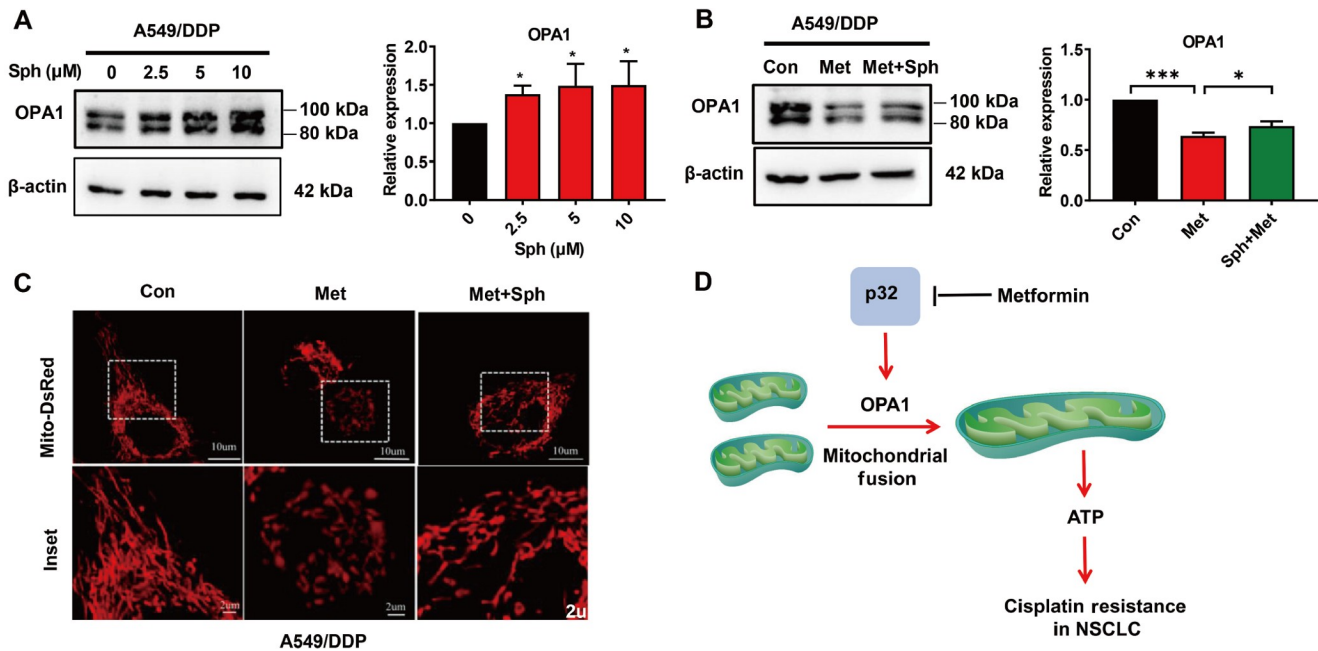
Figure 6. p32 activator abrogates the inhibitory effect of metformin on mitochondrial dynamics (A,B) Effect of D-erythro-Sphingosine on metformin-induced downregulation of OPA1 in A549/DDP cells. After cells were treated with metformin or metformin and D-erythro-Sphingosine for 24 h, whole cell lysates were collected for western blot analysis to detect the expression of OPA1. β-Actin was used as a loading control. *$P < 0.05$, ***$P < 0.001$. (C) Effect of D-erythro-Sphingosine on metformin-induced mitochondrial fission in A549/DDP cells. Cells were transfected with pDsRed2-Mito and treated with metformin for 24 h or in combination with D-erythro-Sphingosine. The fluorescence signal of pDsRed2-Mito was used to label mitochondria and delineate mitochondrial morphology. (D) Schematic diagram illustrating the mechanism of metformin in preventing cisplatin resistance in NSCLC.

dynamics and cisplatin resistance in ovarian cancer, as revealed by elevated mitochondrial fusion in cisplatin-resistant SKOV3/DDP ovarian cancer cells [28]. In addition, upregulation of mitochondrial fusion protein OPA1 expression and downregulation of mitochondrial fission protein Drp1 expression with increased mitochondrial fusion were observed in cisplatin-resistant NSCLC cells in the current study. Furthermore, cisplatin stress also upregulated the expression of OPA1 in NSCLC cells. All these results suggest that OPA1-mediated mitochondrial fusion may be involved in the acquired cisplatin resistance in NSCLC.

As major organelles for ATP synthesis, mitochondria contribute significantly to the cellular energy landscape [29]. Contrary to our previous findings that ATP production was lower in cisplatin-resistant ovarian cancer cells [28], here we found that ATP level was higher in A549/DDP cells than in A549 cells. Inhibition of mitochondrial fusion by *p32* knockdown disrupted mitochondrial membrane potential and significantly decreased intracellular ATP level. These results align with previous studies proposing that high ATP production could facilitate drug resistance in cancer [30]. One mechanism of resistance to cisplatin involves the energy-dependent efflux of the drug out of cancer cells. ATP-binding cassette (ABC) transporters are a family of proteins that can pump various substances across the cell membrane, including cisplatin, using energy derived from ATP [31]. As such, elevated ATP generation might increase the efflux of cisplatin out of A549/DDP cells through ABC transporters. In addition, increased ATP production can augment the capacity of cells to repair DNA damage, the primary cytotoxicity of cisplatin, thereby contributing to cisplatin resistance [32]. Although ATP generation in different cancer cells with drug resistance varies, these differences may be attributable to distinct

cellular contexts.

As mentioned above, both p32 and OPA1 were upregulated and promoted mitochondrial fusion in A549/DDP cells. The next question was whether p32 and OPA1 function in parallel or whether one of them functions as an upstream regulator of the other. Previous studies have shown that *p32* knockdown enhances mitochondrial disruption, suggesting that p32 is critical for maintaining mitochondrial morphology [33]. Until recently, only a few studies have explored the specific mechanisms by which p32 regulates mitochondrial morphology. In the current study, we found that *p32* knockdown downregulated OPA1 expression in A549/DDP cells, but knockdown of *OPA1* had no significant effect on the expression of p32. D-erythro-Sphingosine is an effective p32 kinase activator that increases p32 phosphorylation [34]. Intriguingly, activating p32 by D-erythro-Sphingosine also effectively upregulated the expression of OPA1. These results suggest that p32 acts as an upstream regulator and promotes mitochondrial fusion by upregulating OPA1 expression.

Metformin, a biguanide extracted from *Galega officinalis*, is the first-line oral antidiabetic drug for type 2 diabetes. In the current study, metformin was found to resensitize A549/DDP cells to cisplatin. The role of metformin in cisplatin resistance is contentious across various cancers. While metformin alleviates cisplatin resistance in triple-negative breast cancer by facilitating RAD51 degradation [35], it has also been reported to promote cisplatin resistance in gastric cancer through PINK1/Parkin-mediated mitophagy [36]. Recent reports suggest that metformin improves the overall survival of lung cancer patients [37,38]. Although some previous studies suggest that the antineoplastic effect of metformin is closely related to mitochondrial function [39,40], it is still unclear

whether metformin sensitizes NSCLC to cisplatin by regulating mitochondrial dynamics. In the current study, we tested the effect of metformin on the p32/OPA1 axis, and found that metformin effectively downregulated the expressions of p32 and OPA1 in A549/DDP cells, stimulated mitochondrial fission, disrupted mitochondrial membrane potential, and inhibited intracellular ATP generation. In addition, D-erythro-Sphingosine alleviated the metformin-induced downregulation of OPA1 and mitochondrial fission. Moreover, metformin treatment also sensitized A549/DDP cells to a cisplatin-induced decrease in cell viability. These results suggest that metformin sensitizes resistant NSCLC cells to cisplatin by suppressing p32/OPA1 axis-mediated mitochondrial fusion.

In summary, knockdown of *p32* inhibits OPA1 expression, mitochondrial fusion and ATP production and reduces cisplatin tolerance in NSCLC cells, and D-erythro-Sphingosine upregulates OPA1 expression and mitochondrial fusion. Inhibition of the p32/OPA1 axis by metformin resensitizes A549/DDP cells to cisplatin (Figure 6D). These findings indicate that p32/OPA1 axis-mediated mitochondrial dynamics contributes to the acquired cisplatin resistance in NSCLC and that metformin resensitizes NSCLC to cisplatin, suggesting that targeting p32 and mitochondrial dynamics is an effective strategy for the prevention of cisplatin resistance.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biphysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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