Metformin inhibits the growth of ovarian cancer cells by promoting the Parkin-induced p53 ubiquitination

Running title: <u>Role</u> of metformin in ovarian cancer cells

Xiaojia Min [1, *]**, Tingting Zhang** [2]**, Ying Lin** [1]**, Bo Wang** [1]**, Kean Zhu** [1]

[1]Department of Gynecology, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), Changsha 410002, P.R. China

[2] Department of Gynecology, The Second Xiangya Hospital of Central South University, Changsha 410002, P.R. China

* **Correspondence to:** Xiaojia Min, Department of Gynecology, Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University), No. 61, Jiefang West Road, Changsha 410002, Hunan Province, P.R. China

E-mail: minxiaojia@hunnu.edu.cn

Tel.: +86-13875870384

Abstract

Ovarian cancer is the most lethal diseases among women. The chemo-resistance has been a big challenge for the cancer treatment. It has been reported that metformin may inhibit ovarian cancer and is able to impede the development of drug resistance, but the molecular mechanisms remain elusive. In this study, we explored the molecular roles of metformin in Parkin expression and p53 ubiquitination in chemo-resistant ovarian cancer cells. Firstly, ovarian cancer and chemo-resistant ovarian cancer cells were selected for determining the expression of Parkin, p53, and p53 signaling pathway-related factors. Then the cell proliferation and viability after loss- and gain-of-function assays were measured. Besides, immunoprecipitation (IP) was used to determine the interactions between Parkin and p53, and the ubiquitination level of p53 was measured using *in vitro* ubiquitination assay. Finally, the degradation of p53 proteasome regulated by Parkin was monitored using the MG132 proteasome inhibitor. We found that metformin significantly inhibited the growth of ovarian cancer parental cells and chemo-resistant cells, and metformin promoted Parkin expression in chemo-resistant cells. Further, up-regulated Parkin expression promoted the ubiquitination and degradation of p53, and metformin inhibited the expression of p53 to suppress the proliferation of chemo-resistant ovarian cancer cells. Mechanistically, metformin could inhibit the growth of ovarian cancer cells by promoting the Parkin-induced p53 ubiquitination. Altogether, our study demonstrated an inhibitory role of metformin in the growth of chemo-resistant cancer cells through promoting the Parkin-induced p53 ubiquitination, which provides a novel mechanism of metformin for treating ovarian cancer.

Keywords

Introduction

Ovarian cancer is one of the most prevalent gynecological malignant tumors [1]. The incidence of ovarian cancer differs among countries: the highest incidence rates are shown in the developed regions, while the lowest rates observed in African and Asian parts [2]. Due to the limitations of therapeutic treatments, there's rapid development and frequent cancer recurrence for this disease, making the outcome of ovarian cancer treatment comparatively poor [3]. Moreover, increasing number of patients with relapse in one to two years under different circumstances gradually develop resistance to the chemotherapies [4]. Therefore, it is urgent to explore the underlying molecular mechanisms of progression of chemo-resistance of ovarian cancer and to identify novel targets for the therapy of ovarian cancer. Recently, a study has indicated that metformin may have an anti-cancer effect on ovarian cancer through inhibition of the ovarian cancer cell proliferation [5].

Metformin (1,1-dimethylbiguanide) is one of the most effective treatment to patients with type 2 diabetes mellitus (T2DM), making itself the most commonly prescribed therapy for T2DM [6]. Many studies have reported a survival benefit with metformin use in many cancers. For example, a study found that those patients with non-small cell lung cancer (stage IV) who received metformin showed notably higher survival rate [7]. Additionally, the previous study has demonstrated that metformin suppresses the expression of ovarian cancer-related genes, cell migration and proliferation of ovarian cancer cells, which suggests that it can be a therapeutic target for ovarian cancer [8]. However, the role of metformin mechanism in the chemo-resistance in ovarian cancer is rarely discussed and needs to be further explored. Among the various ubiquitin ligase, the impacts of Parkin on different diseases have been recently revealed. For instance, as an E3 ubiquitin ligase, Parkin is involved in the cell biological function of keloids [9]. In Parkinson's disease, Parkin is a tumor suppressor that affects necroptosis and inflammation [10]. Moreover, the chemo-resistance is

controlled by Parkin in mitophagy [11]. However, there are few studies on the relationship between Parkin and ovarian cancer. Furthermore, as one of the most studied oncogenes, p53 mutations are commonly found and its expression is altered in nearly a half of human malignant tumors [12]. It is reported that the aggregation of p53 exerts crucial function in tumor progression and cell survival, which is associated with the chemo-resistance reversal among ovarian cancer patients [13], which makes it a critical therapeutic target for cancers.

From all above, we hypothesized that metformin could affect the chemo-resistant ovarian cancer cells by regulating the expression of Parkin as well as the p53 ubiquitination. Therefore, in our study, we explored the correlations among metformin treatment, Parkin and p53, aiming to provide a novel treatment and therapeutic targets for the ovarian cancer.

Materials and methods

Cell treatment

Ovarian cancer parental cell line A2780 and cisplatin-resistant cell line A2780-cis (referred to the website https://www.sigmaaldrich.com/catalog/product/sigma/cb_93112517) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). The cisplatin sensitive IGROV1 cell line was purchased from American Type Culture Collection (American Type Culture Collection, Virginia, USA) [14]. The aforementioned cells were cultured in RPMI-1640 (Gibco Company, Grand Island, NY, USA) medium with 10% Fetal Bovine Serum (FBS) (Gibco Company, Grand Island, NY, USA) and 1% (V/V) penicillin/streptomycin at 37°C, 5% CO_2. Metformin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved into different concentrations by phosphate buffer saline (PBS).

A2780-cis and IGROV1-cis cells were cultured in a 6-well plate, with the concentration of 2×10^5 cells per well. When the cell confluence reached 80%, the cells were transfected according to

Lipofectamine 2000 instructions (11668-019, Invitrogen, Carlsbad, California, USA). The cells were treated with the plasmids of siRNA against Parkin or p53 and their corresponding non-sense RNA negative control (NC). All the plasmids above were purchased from GenePharma Co., Ltd (Shanghai, China). The cells after treatment were cultured at 37°C and 5% CO_2 for 48 h, which was used for subsequent experiments.

Clonogenic assay

Cell proliferation was detected using the clonogenic assay. First, 1.2 % agar was heated, dissolved and then placed in water at 46°C. The prepared ovarian cancer cells were counted and suspended in preheated RPMI1640 medium (40°C) containing 40% fetal calf serum (FCS). A total of 325 μL of the cell suspension (including 1×10^5 cells) was added to each well of the 24-well culture plate, and 50 μL of the sample to be tested was added. A suitable amount of 125 μL preheated agar (1.2%) was mixed with the cell suspension and the sample to be tested in a 24-well plate. The mixture was incubated at 37°C and 5% CO_2 for 8-10 days after coagulation, and then observed under an inverted microscope. Each experiment was repeated three times.

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium (MTT) assay

MTT assay was used to detect the effect of metformin on the cytotoxicity of A2780, A2780-cis, IGROV1, and IGROV1-cis cells. In short, cells (1×10^4 cells / well) were inoculated into a 96-well plate. Afterwards, the cells were exposed to metformin (0, 5, 10 and 20 mM) at 100 μL cells/well for 48 h. Chemo-free medium was added to the control and blank wells, and 20 μL MTT (5mg/mL) was added to each well after treatment. After 4 h, the MTT solution was removed and the insoluble formazan crystals were dissolved in 150 μL dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis,

Mo, USA). Flex-station 3 (Molecular Devices Corporation, CA, USA) was used for optical density measurement. Each experiment was repeated three times.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Different groups of ovarian cancer cell samples were collected and lysed with Trizol reagent (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) to extract total RNA. The quality and concentration of RNA were determined by ultraviolet visible spectrophotometry (ND-1000, NanoDrop Technologies Inc., Wilmington, USA). The extracted 400 ng total RNA was reversely transcribed following the instructions of PrimeScript RT Reagent Kit (Takara Biotechnology Ltd., Dalian, China). Then fluorescence quantitative PCR was performed according to the instructions of SYBR®Premix ExTaqTM II kit (TLI RNaseH plus; Takara Bio Inc., Tokyo, Japan) with complementary DNA (cDNA) as template. The primers were synthesized by Guangzhou RiboBio Co., Ltd. (Guangdong, China) (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference. The relative expression of gene was calculated by $2^{-\Delta\Delta Ct}$ method with each experiment repeated three times.

Western blot analysis

Different groups of ovarian cancer cells were cleaned with PBS, then lysed by cell lysis buffer (c0481, Sigma-Aldrich, St Louis, MO, USA) and incubated at 4℃ for 30 min. The lysate was collected into a 1.5-mL EP tube and centrifuged at 12000 g and 4℃ for 15 min. The protein concentration was measured by the bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology, Shanghai, China). The protein loading buffer was added into the supernatant and boiled for 5 min. Then, the 20 mg protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Billirica, Ma, USA), which was blocked with 5% skimmed milk powder for 1 h at room temperature. The membrane was incubated with Tris-Buffered Saline

Tween-20 (TBST)-diluted primary rabbit antibodies to Parkin (ab77924, 1 : 1000), P53 (ab32389, 1 : 1000), phosphorylated p53 content (ab1431, 1 : 1000), p21 (ab218311, 1 : 1000) and MDM2 (ab170880, 1 : 1000) overnight at 4℃. After washed three times with TBST, the membrane was further incubated with Horseradish Peroxidase (HRP) labeled secondary goat anti-rabbit antibody (ab205718, 1 : 10000) and secondary goat anti-mouse antibody (ab205719, 1 : 10000) at room temperature for 1 h. The image was developed by enhanced chemiluminescence (Bio-Rad laboratories, Inc., Hercules, California, USA) with the rabbit antibody to GAPDH (ab37168, 1 : 1000) as the internal reference. All the antibodies above were purchased from Abcam Inc. (Cambridge, UK). The gray value of each band was analyzed by gel image analysis software Image J.

Immunoprecipitation (IP)

The cells were lysed in the EBC lysis buffer. The soluble supernatant was mixed with polyclonal anti-p53 antibody and incubated overnight at 4℃. Protein A/G beads were then added to the reaction mixture. After washing the immune complexes, the protein was re-suspended in SDS sample buffer and separated by SDS-PAGE. The monoclonal rabbit antibody against Parkin (ab77924, 1 : 100, Abcam Inc., Cambridge, UK) was analyzed by western blot analysis. The experiment details were referred to the methods established before [15].

***In vitro* ubiquitination assay**

In vitro ubiquitination assay was carried out using the commercially available kit (Boston Biochem, Inc., Cambridge, Ma, USA) according to the manufacturer's instructions. The specific operation methods were referred to previous study [15]. Briefly, the HEK293 cell was transfected with the plasmids Ha-ubiquitin (2 μg), Flag-p53 (1 μg) and myc-Parkin (1 μg) using the Lipofectamine 3000. After 48 h, the cells were collected for western blot analysis and ubiquitination detection. The protein was analyzed and eluted by western blot analysis with p53 monoclonal antibody.

Statistical analysis

Statistical analysis was conducted using SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. The unpaired data conforming to normal distribution and homogeneous variance between two groups were analyzed by unpaired *t*-test. Comparisons among multiple groups were conducted using the one-way analysis of variance (ANOVA) with Tukey's post hoc test used. Data comparison among groups were analyzed by the repeated measures ANOVA followed by Bonferroni's post hoc test. A value of $p < 0.05$ was considered as statistically significant.

Results

Metformin inhibits the growth of ovarian cancer parental cells and chemo-resistant cells

In order to explore the effect of metformin on the chemo-resistance of ovarian cancer cells, the ovarian cancer parental cells A2780 and IGROV1 and chemo-resistant cell A2780-cis, IGROV1-cis were treated with different concentrations (0, 5, 10, 20 mM) of metformin, respectively. The proliferation of the aforementioned cells treated with different concentrations of metformin was measured by the clonogenic assay. The results showed that the growth of cells was inhibited by metformin in a dose-dependent manner (Figure 1A and 1B). Subsequently, MTT assay was used to measure the viability of cells treated with different concentrations of metformin. The results depicted that the increase of metformin concentration led to decreased cell viability, and the viability of chemo-sensitive cells was significantly lower than that of chemo-resistant cells (Figure 1C). The aforementioned findings revealed that metformin significantly inhibited the growth of both chemo-sensitive and chemo-resistant ovarian cancer cells.

Metformin inhibits the cell growth though upregulation of the expression of Parkin in chemo-resistant ovarian cancer cells

To investigate the mechanisms of ovarian cancer cell resistance to metformin, the expression of Parkin in parental cells A2780 and IGROV1 as well as chemo-resistant cells A2780-cis and IGROV1-cis after treated with different concentrations (0, 5, 10, 20mM) of metformin was measured by Western blot analysis. We found that the Parkin expression in A2780-cis and IGROV1-cis cells after treatment of 20 mM metformin was significantly increased (Figure 2A). To further dissect the role of Parkin in the effects of metformin on ovarian cancer cells, we used siRNA to knock down the expression of Parkin in cells. The mRNA and the protein expression of Parkin were assessed by RT-qPCR and western blot analysis after silencing Parkin expression in A2780-cis and IGROV1-cis cells. Our results confirmed that mRNA and protein expression of Parkin was notably decreased after siRNA treatment (Figure 2B). Clonogenic assay was used to detect the effect of metformin (20 mM) on the proliferation of A2780-cis and IGROV1-cis cells after Parkin knockdown. siRNA against Parkin treatment significantly promoted the effect of metformin on proliferation of A2780-cis and IGROV1-cis cells (Figure 2C and 2D). To further explore the effect of metformin on A2780-cis and IGROV1-cis cells after silencing Parkin, MTT assay was used to detect the changes of viability and IC50 value of cells after Parkin knockdown with the results displaying that the viability of cells with si-Parkin was increased significantly after treating with metformin (Figure 2E). These results indicated that metformin promoted the expression of Parkin and suppressed cell viability.

Metformin promotes the Parkin-mediated ubiquitination of p53 in chemo-resistant ovarian cancer cells

Given that Parkin is an important E2 ubiquitin ligase, we aimed to determine whether metformin promoted the ubiquitination and degradation of p53 by upregulating the expression of Parkin. Therefore we detected the binding of Parkin to p53 in A2780-cis and IGROV1-cis cells by IP assay. The results demonstrated the interaction between Parkin and p53 in A2780-cis and IGROV1-cis cells (Figure 3A). Following after, the ubiquitination of p53 in cells treated with siRNA against

Parkin was measured with the results indicated that the ubiquitination of p53 after siRNA against Parkin treatment was significantly decreased, compared to that in cells transfected with siRNA NC (Figure 3B). To further explore the interactions between Parkin and p53, we took advantage of the HEK293 cells and overexpression of tagged target proteins. After HEK293 cells were transfected with the plasmids of myc labeled Parkin and hydroxyapatite (HA) labeled ubiquitin, myc and HA tags of cell extracts were analyzed by western blot analysis. Parkin expression was found in the transfected cell extract with HA tag to increase the protein ubiquitination. However, the expression of Parkin was not observed in the extracts without HA labeled ubiquitin (Figure 3C). And then the effect of Parkin on the ubiquitination of p53 was detected *in vitro*. The results showed that Parkin could promote the ubiquitination of p53 (Figure 3D). The expression of Parkin increased after treating with 20 mM metformin and 10 μM MG132, which promoted the ubiquitination and degradation of p53 (Figure 3E). These results suggested that the ubiquitination of p53 was mediated by Parkin in ovarian cancer chemo-resistant cells, and the degradation of p53 was promoted by metformin.

Metformin suppresses the proliferation of chemo-resistant ovarian cancer cells through downregulation of the expression of p53

To further explore whether p53 is involved in the chemo-resistance of ovarian cancer cells, we treated A2780-cis and IGROV1-cis cells with different concentrations of metformin to measure the related protein expression of p53 and p53 pathway-related factors (phosphorylated p53, p21 and MDM2). The results showed that the expression of p53 and p53 pathway-related factors were decreased significantly by metformin treatment in a dose-dependent manner (Figure 4A). Given the effects of metformin treatment on p53 signaling, we'd like to know whether metformin affected the chemo-resistance of ovarian cancer cells through Parkin/p53 signaling pathway. Thus we silenced p53 expression in A2780-cis and IGROV1-cis cells with siRNA with the silencing efficacy confirmed by RT-qPCR and western blot analysis that the mRNA and protein expression of p53

were notably reduced after siRNA against p53 treatment (Figure 4B). Then with silenced p53 expression, we treated the cells with 20 mM metformin and measured the expression of p53. Surprisingly, the expression of p53 was further reduced with metformin treatment (Figure 4B). Then clonogenic assay was used to detect the proliferation of cells in different groups. Silencing p53 significantly inhibited the proliferation of cells which was further inhibited by metformin treatment (Figure 4C). MTT assay was used to detect the cell viability in different groups. The results demonstrated that silencing p53 notably reduced the effect of metformin on cell viability (Figure 4D). Additionally, Western blot analysis was performed to measure the protein expression of p53 pathway in different groups of cells. The results revealed that silencing p53 suppressed the expression of p53 pathway-related factors in cells. Besides, cells with siRNA against p53 after metformin treatment showed further inhibited expression of p53 pathway-related factors, and increased Parkin expression (Figure 4E). These results indicated that through Parkin/p53 pathway, metformin inhibited the proliferation of ovarian cancer chemo-resistant cells.

Discussion

The etiology and molecular biology of the malignancy of ovarian cancer remain elusive [16]. With the progress of ovarian cancer, the cell biological properties of the cancer cells becomes more and more complicated, and most patients who previously positively respond to chemotherapy may die of recurrent chemo-resistant tumors. This indicates that the chemo-resistance is dynamically developed and hard for patients to overcome [13]. Therefore, to better understand the molecular mechanisms of chemo-resistance and discovery of therapeutic targets is critical for the treatment of ovarian cancer cells. As a FDA approved drug for treating T2DM, metformin has also been reported to play an inhibitory role in the development of cancer stem cells and epithelial ovarian cancer cell [17]. However, the mechanism remains unclear. Our study explored the role of metformin in ovarian cancer chemo-resistant cells and we found that metformin inhibits the growth of ovarian cancer parental cells and chemo-resistant cells through acting on Parkin and p53 ubiquitination.

As we have shown, metformin significantly suppressed the growth of chemo-resistant ovarian cancer cells. What's more, many other studies have found that metformin is a potential treatment for diverse cancers. For example, a previous study reveals that metformin up-regulates miR-708-5p expression and promotes the cell apoptosis in prostate cancer [18]. The correlation between metformin and ovarian cancer has been explored. Metformin affects the ovarian cancer cell metabolism, prevents the growth of tumors and strengthens the sensitivity to chemotherapies both *in vivo* and *in vitro*, indicating that metformin could be a drug for the treatment of ovarian cancer [5]. Consistent with our findings, a previous study has demonstrated the inhibitory effect of metformin on ovarian cancer cell migration and proliferation as well as the expression of cancer-related genes of adipocyte-mediated cell [8].

The growth of cancer cells was restrained by the regulation of Parkin by metformin. As a tumor suppressor, Parkin also plays an inhibitory role in breast cancer metastasis [19]. A previous study shows that Parkin expression was downregulated in the cells and tissues of breast cancer, suggesting that Parkin is involved in the development of breast cancer [20]. Previous studies also revealed the correlation between metformin and Parkin. For example, metformin could significantly increase the expression of Parkin and promote the interaction between Parkin and hypoxia-inducible factor-1α, indicating that the cooperation of metformin and Parkin can be a therapeutic agent for keloids [9]. This finding is exactly consistent with our results that metformin promoted the expression of Parkin. Moreover, our study also showed that silencing Parkin by siRNA could increase the proliferation and viability of chemo-resistant A2780-cis and IGROV1-cis cells. Correspondingly, previous study unraveled that the loss of Parkin accelerated the progression of cancers including lung cancer and breast cancer [21]. Taken together, Parkin plays critical roles in multiple cancers.

Additionally, we also found that metformin treatment decreased the expression of p53 and p53 pathway-related factors in a dose-dependent manner. Qiang *et al.* reported that metformin could inhibit the activation of MDM2 to inhibit malignant phenotypes of endometrial cancer cells [22].

Importantly, targeting p53-MDM2 pathway is demonstrated as the goal of preventing and treating cancers in the current stage [23]. Interestingly, our study further illustrated the relation between Parkin and p53 ubiquitination. It has been reported that Parkin interacts with p53, which leads to the loss of p53 function in rheumatoid arthritis [15]. A previous study has demonstrated that the binding of metformin and 2-deoxyglucose can promote the p53-dependent apoptosis in prostate cancer [2224]. Moreover, in human ovarian cancer cells, p53 is associated with Parkin-like cytoplasmic protein to promote the chemo-resistance [2325]. Similar as Parkin, murine double minute, as an E3 ligase, exerts the same promoting function to p53 ubiquitination and degradation [2426]. These findings highlight the ubiquitination of p53 by various E3 ubiquitin ligase like Parkin in the development of cancers.

In conclusion, we find that metformin can upregulate the expression of Parkin and promote p53 ubiquitination to inhibit the growth of ovarian cancer parental cells and chemo-resistant cells (Figure 5). These findings revealed that metformin could serve as a therapeutic agent for ovarian cancer. However, more studies remains needed to further explore the mechanisms and overcome the limitations of this study. For instance, the *in vivo* experiments are required in future study. Besides, the interactions among metformin, Parkin and p53 remain to be more identified in ovarian cancer. We will further explore the underlying rules governing metformin-Parkin interaction in the future.

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Competing interests

The authors declare that they have no competing interests.

Author Contribution

Xiaojia Min and Bo Wang wrote the paper and conceived and designed the experiments; Tingting Zhang analyzed the data; Ying Lin and Kean Zhu collected and provided the sample for this study. All authors have read and approved the final submitted manuscript.

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Figure legends

Figure 1 Metformin suppresses the growth of ovarian cancer cells. A, The growth of ovarian cancer parental cells A2780, IGROV1 and resistant cells A2780-cis, IGROV1-cis treated with different concentrations of metformin (0, 5, 10 and 20 mM) detected by clonogenic assay. B, The number of colonies after treatment with metformin of different concentrations for 48 h counted by hematometer. C, Cell viability assessed by MTT assay. *, $p < 0.05$ vs. the chemo-resistant cells A2780-cis, IGROV1-cis treated with different concentrations of metformin. Comparisons among multiple groups were analyzed using the one-way analysis of variance (ANOVA) with Tukey's post hoc test. Comparison among groups were analyzed by the repeated measures ANOVA followed by Bonferroni's post hoc test.
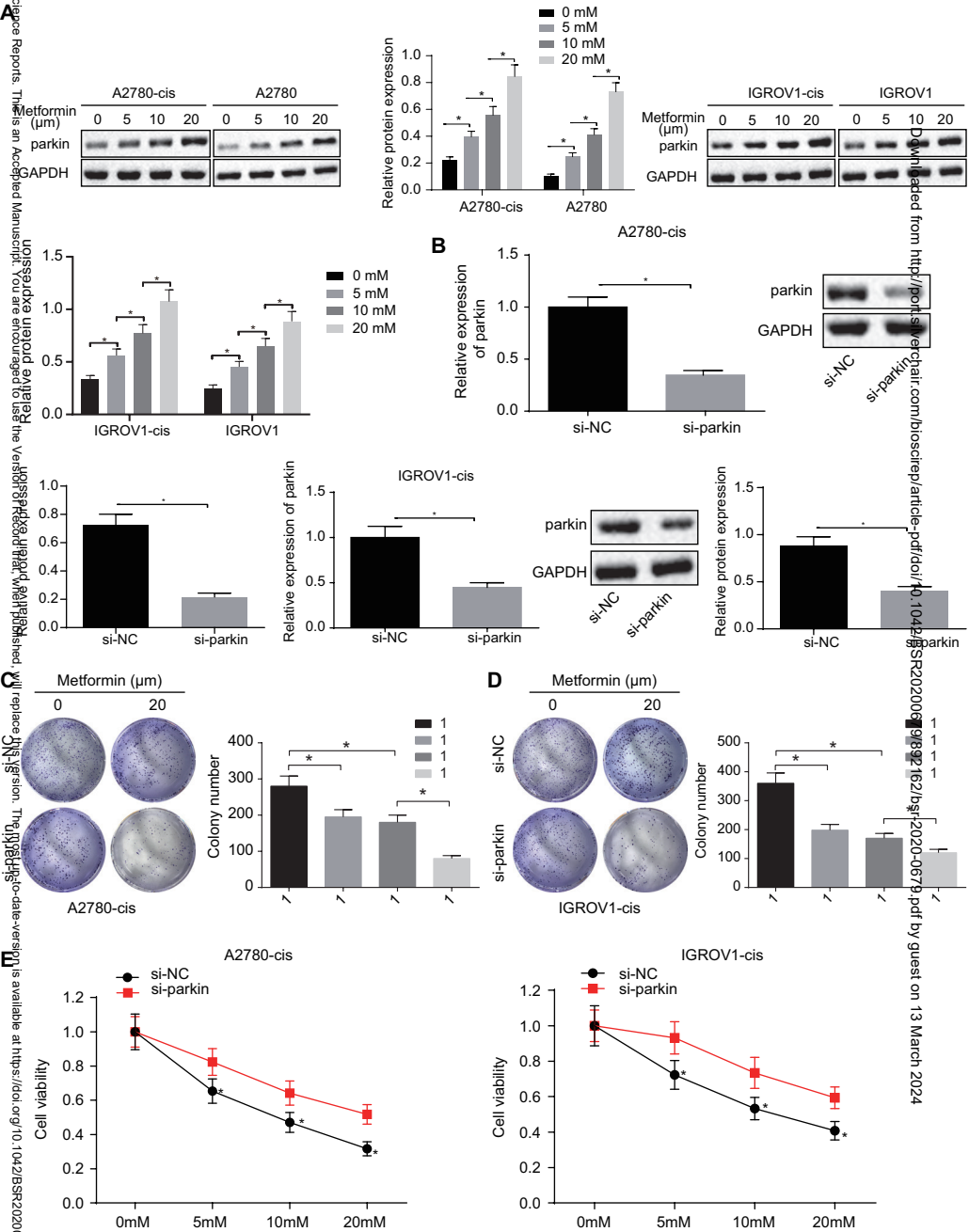
Figure 2 Metformin promotes the expression of Parkin and inhibits cell viability. A, Western blot analysis showing the expression of Parkin in parental and chemo-resistant ovarian cancer cells after treating with metformin of different concentrations. B, The mRNA and protein expression of Parkin measured by RT-qPCR and western blot analysis. C and D, The growth of A2780-cis and IGROV1-cis cells treated with 20 mM metformin and siRNA against Parkin measured by clonogenic assay. E, The viability of differently treated cells assessed by MTT assay. *, $p < 0.05$ vs. the cells treated with siRNA against Parkin. The data conforming to normal distribution and homogeneous variance between two groups were analyzed by unpaired *t*-test. Comparisons among multiple groups were analyzed using the one-way ANOVA with Tukey's post hoc test. Data comparison among groups were analyzed by the repeated measures ANOVA followed by Bonferroni's post hoc test.

Figure 3 Metformin increases the Parkin-mediated ubiquitination of p53 in chemo-resistant ovarian cancer cells. A, The interaction of Parkin and p53 in A2780-cis and IGROV1-cis cells detected by IP assay. B, The ubiquitination of p53 in A2780-cis and IGROV1-cis cells after treating with silencing Parkin was measured. C, The ubiquitination of p53 by Parkin assessed *in vitro*: the ubiquitination of p53 measured by western blot analysis after HEK293 cells transfected with the

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plasmids of myc labeled Parkin and HA labeled ubiquitin. D, The ubiquitination of p53 by Parkin detected *in vitro*, and the reaction mixture analyzed by western blot analysis. E, The ubiquitination of p53 *in vivo* treated with 20mM metformin. The cell lysate immunoprecipitated with anti-ubiquitin antibody, and designated antibody analyzed by western blot analysis. Data between two groups were analyzed by unpaired *t*-test.

Figure 4 Metformin inhibited the proliferation of chemo-resistant ovarian cancer cells. A, The protein expression of p53 and p53 pathway-related factors (phosphorylated p53, p21 and MDM2) in ovarian cancer resistant cells with different concentrations of metformin measured by western blot analysis. B, The mRNA and protein expression levels of p53 assessed by RT-qPCR and western blot analysis. C, The growth of A2780-cis and IGROV1-cis cells treated with 20 mM metformin after silencing p53 measured by clonogenic assay. D, The viability of different treated cells measured by MTT assay. E, The expression of p53 pathway-related factors in the cells after silencing p53 measured by western blot analysis. *, $p < 0.05$ vs. the cells treated with si-NC. Unpaired *t*-test was used for testing the difference between data conformed to restrained for normal distribution and homogeneous variance. Comparisons among multiple groups were analyzed using the one-way analysis of variance (ANOVA) with Tukey's post hoc test used. Data comparison among groups were analyzed by the repeated measures ANOVA followed by Bonferroni's post hoc test.

Figure 5 Molecular mechanism of the effects of metformin on ovarian cancer cells. Metformin can inhibit the chemo-resistant ovarian cancer by upregulating the expression of Parkin and promoting p53 ubiquitination.

Table 1 Primer sequences for RT-qPCR

Name	Sequence （5'-3'）
Parkin	F: 5'-CCTGCTGTTCTC TCGCGC -3'
	R: 5'-GGT CAGAGA CCCGGACCC -3'
p53	F: 5'-TGCTGAGTATCTGGACGACA -3'
	R: 5'-CAGCGTGATGATGGTAAGG -3'
GAPDH	F: 5'-ACAGTCAGCCGCATCTTCTT-3'
	R: 5'-GACAAGCTTCCCGTTCTCAG -3'

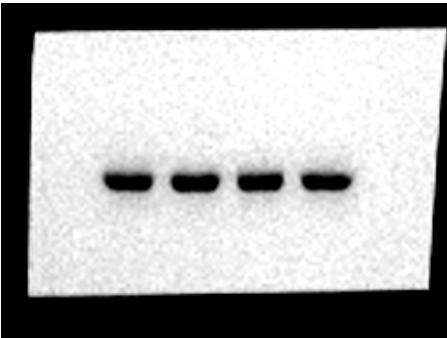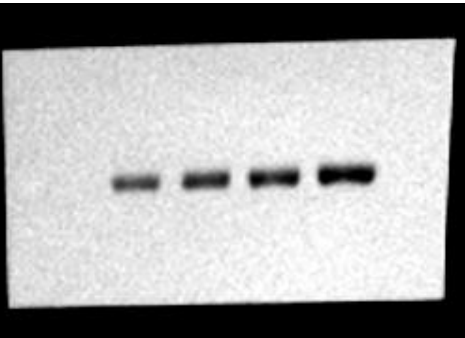Notes: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse
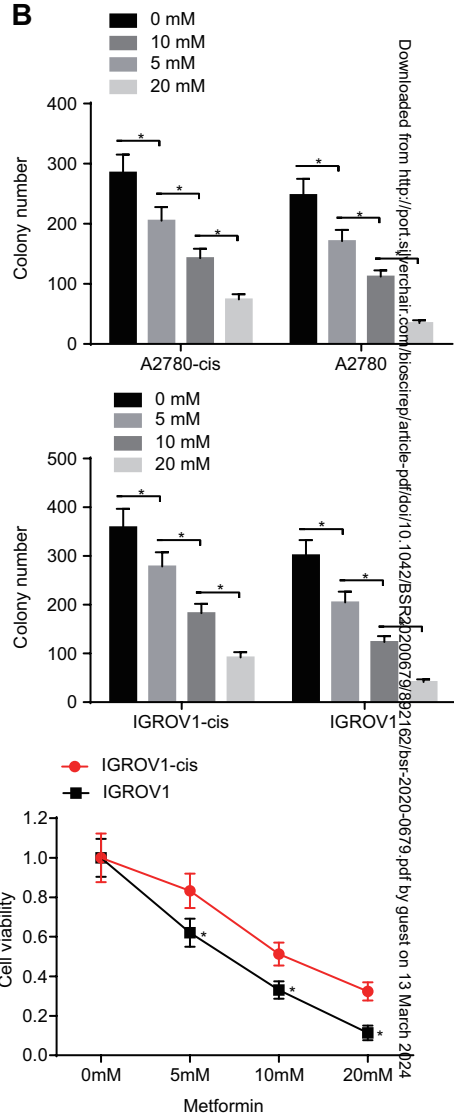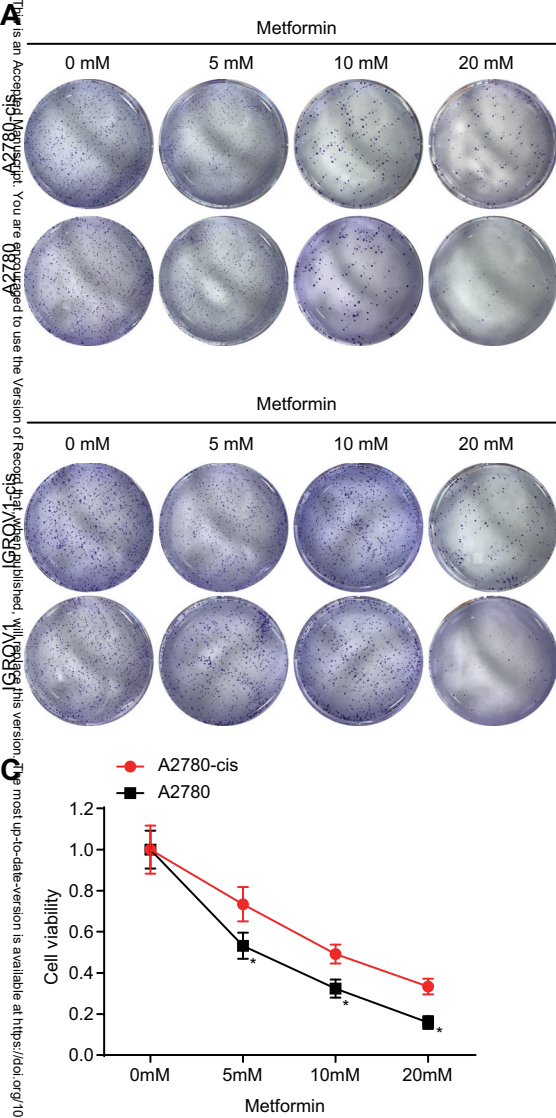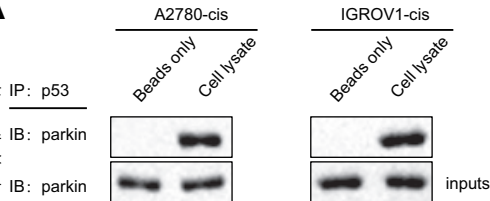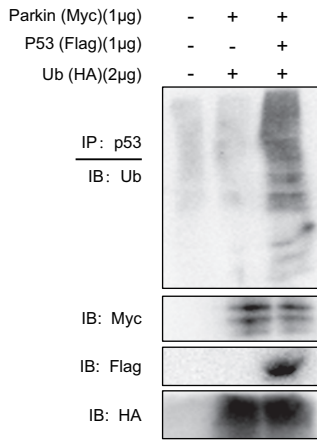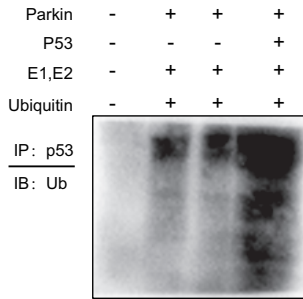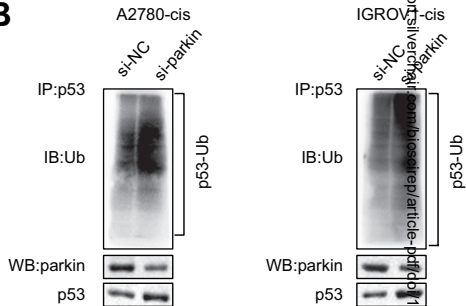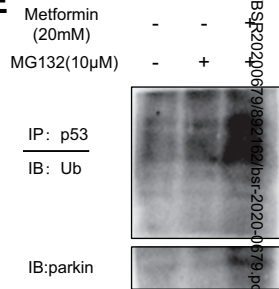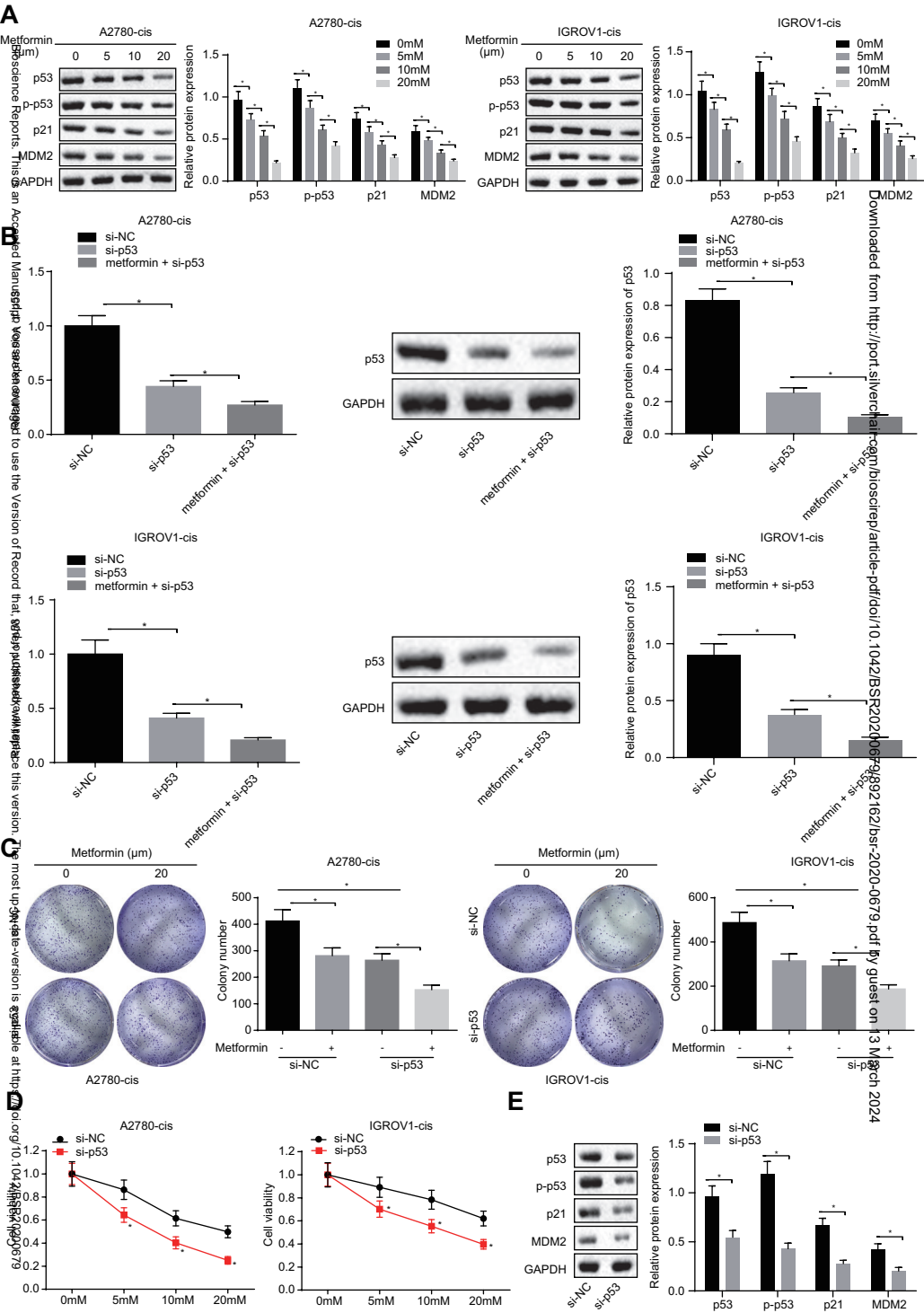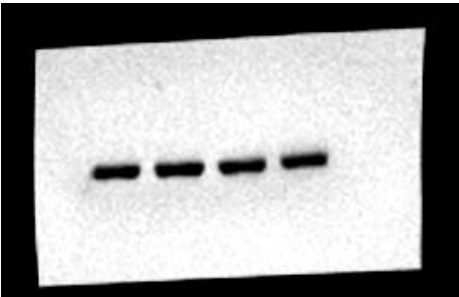
Figure 2A

A2780 (GAPDH)
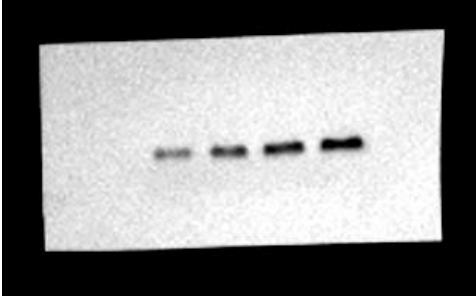
A2780 (parkin)

A2780-cis(GAPDH)

A2780-cis(parkin)
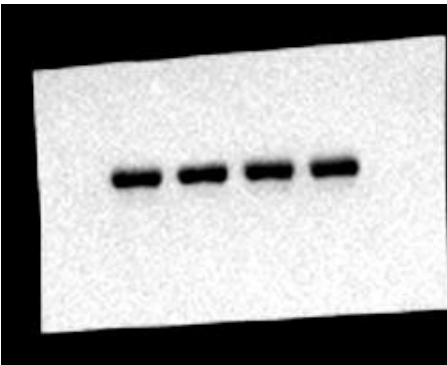
IGROV1 (GAPDH)
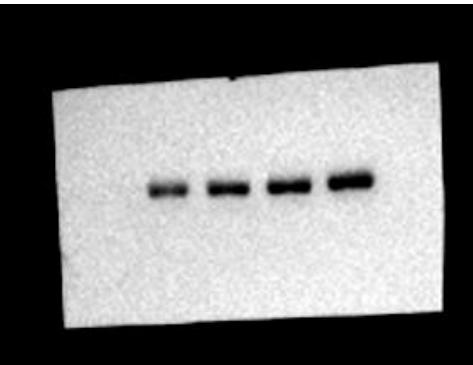
IGROV1 (parkin)
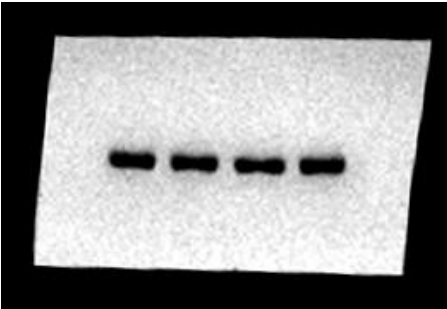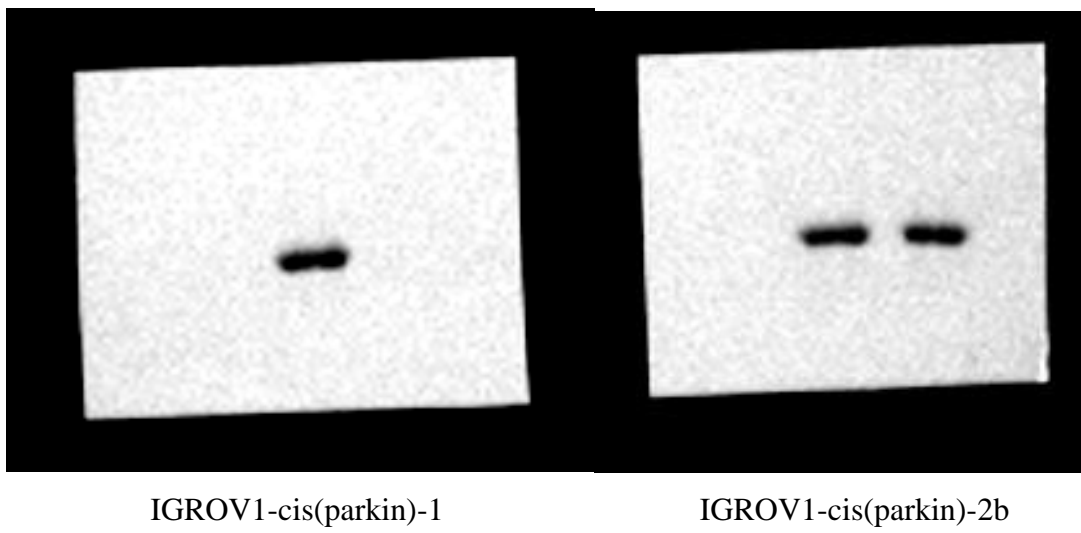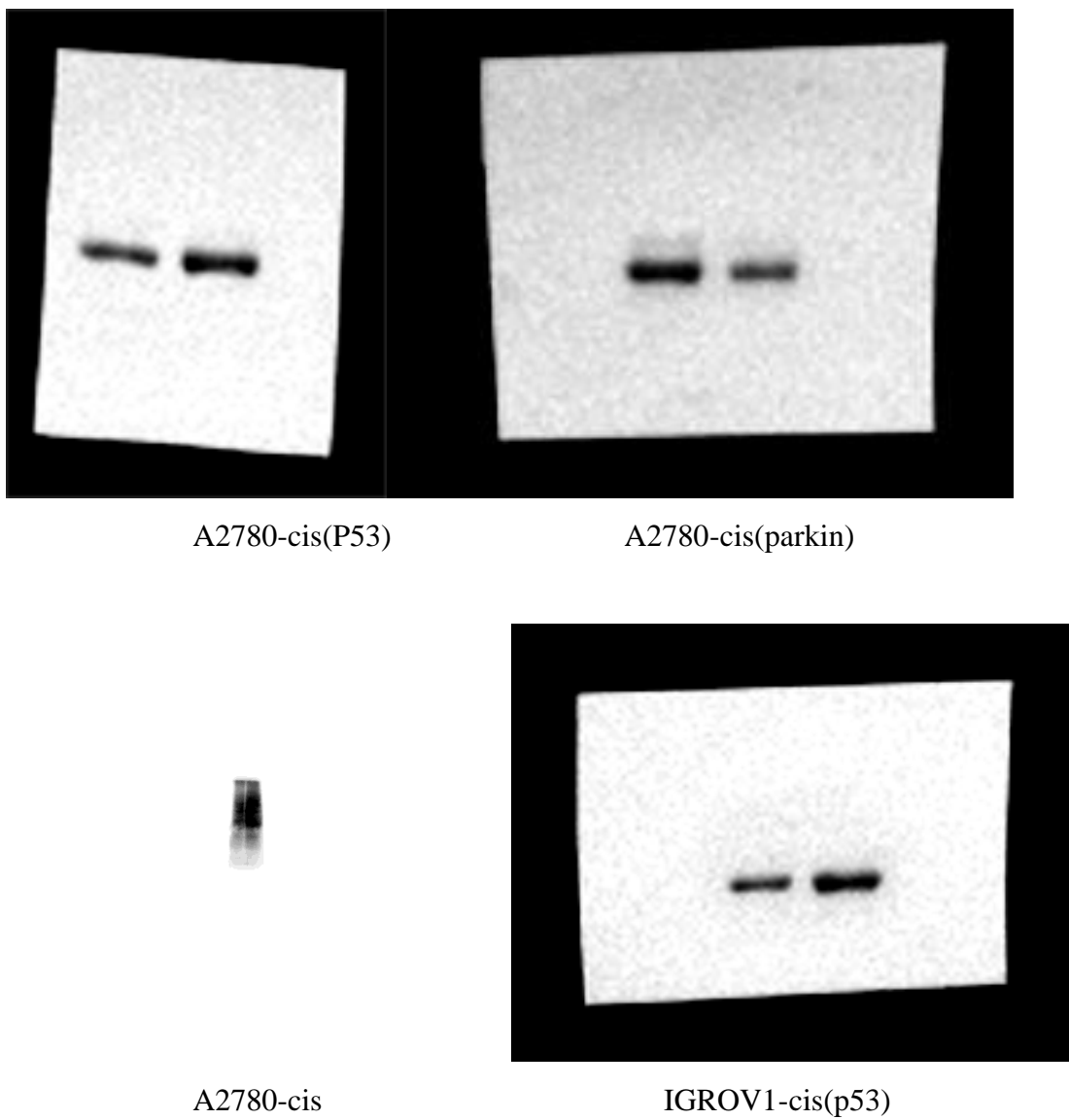
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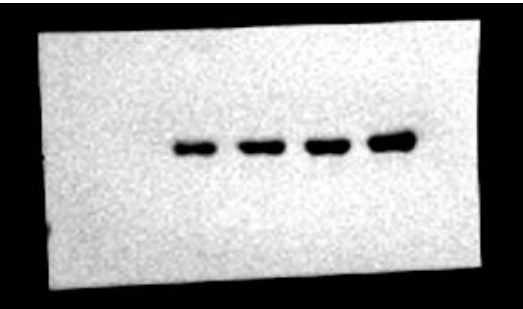
IGROV1-cis(parkin)

Figure 2B

A2780 (GAPDH)　　　　　　　　　　A2780 (parkin)

A2780-cis(GAPDH)　　　　　　　　A2780-cis(parkin)

Figure 3A

A2780-cis(parkin)-1　　　　　　　A2780-cis(parkin)-2

IGROV1-cis(parkin)-1   IGROV1-cis(parkin)-2b

Figure 3B

A2780-cis(P53)   A2780-cis(parkin)
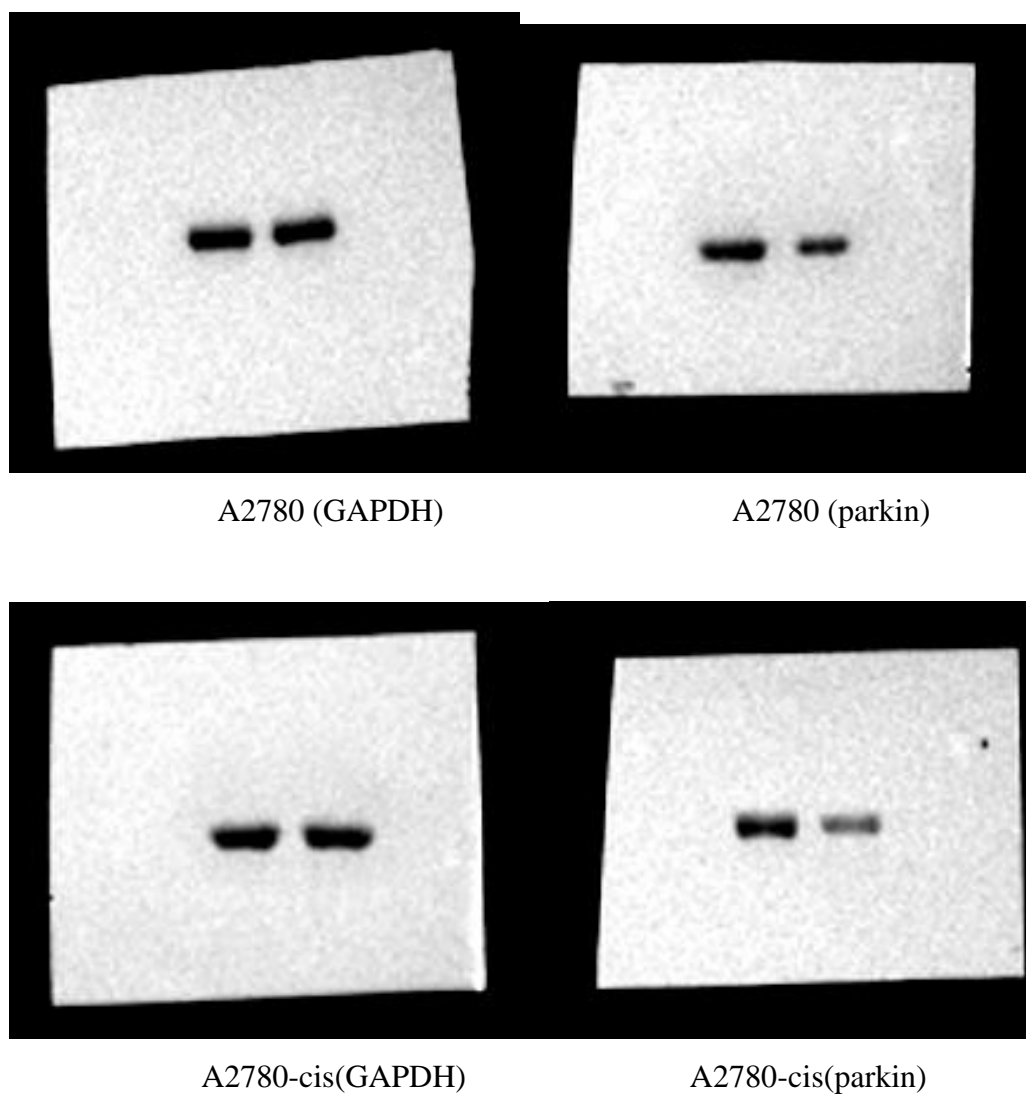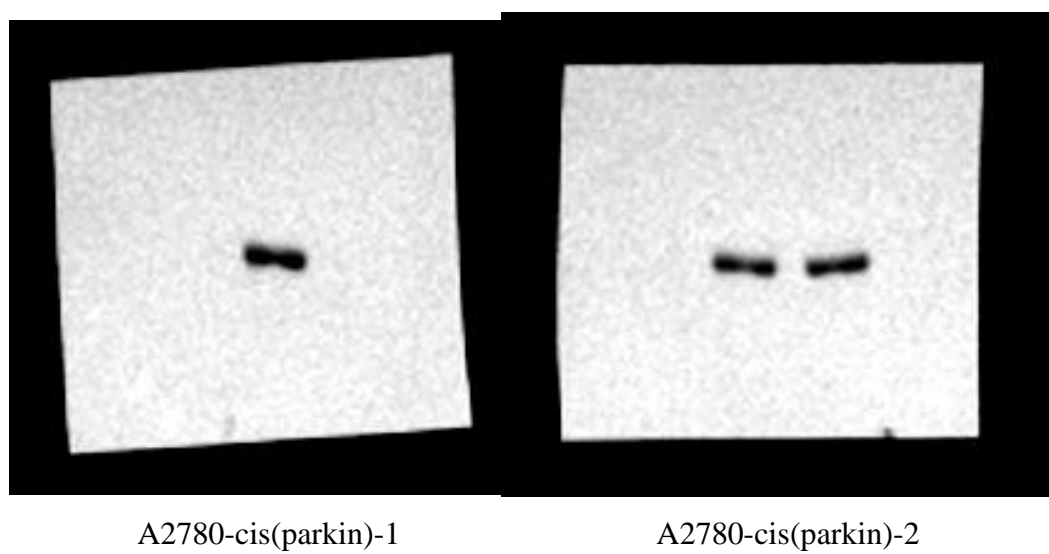
A2780-cis   IGROV1-cis(p53)
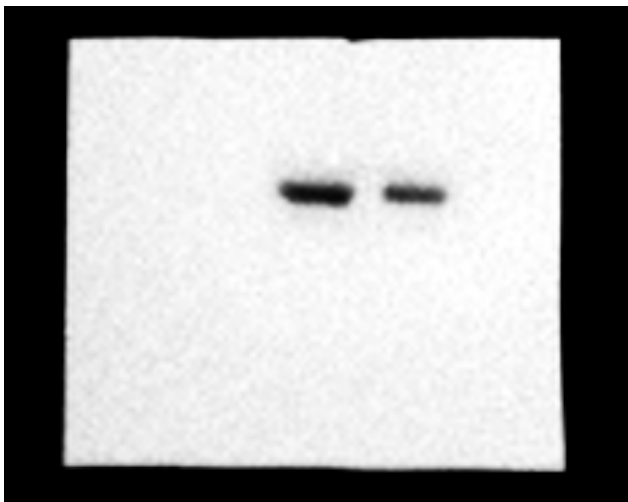
IGROV1-cis(parkin)   IGROV1-cis

Figure 3C
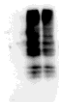

Figure 3C-IB: Flag   Figure 3C-IB:HA

Figure 3C-IB:MYC   Figure 3C-IP:p53-IB：Ub

Figure 3D

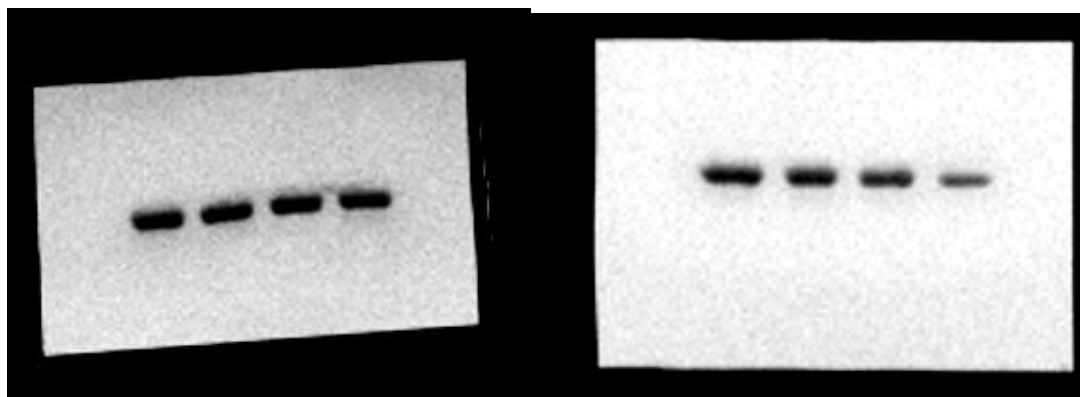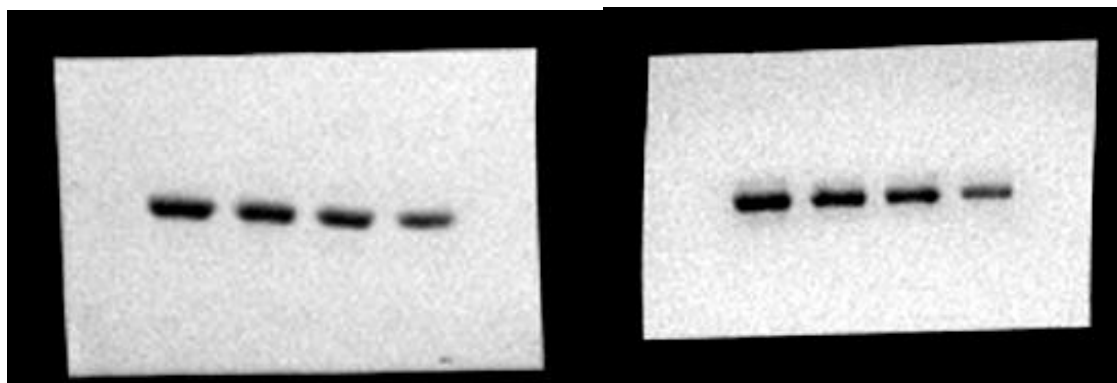**Figure 3E**

Figure 3E-IB: parkin   Figure 3E-IP: p53-IB：Ub

Figure 4A

A2780-cis(GAPDH)   A2780-cis(MDM2)
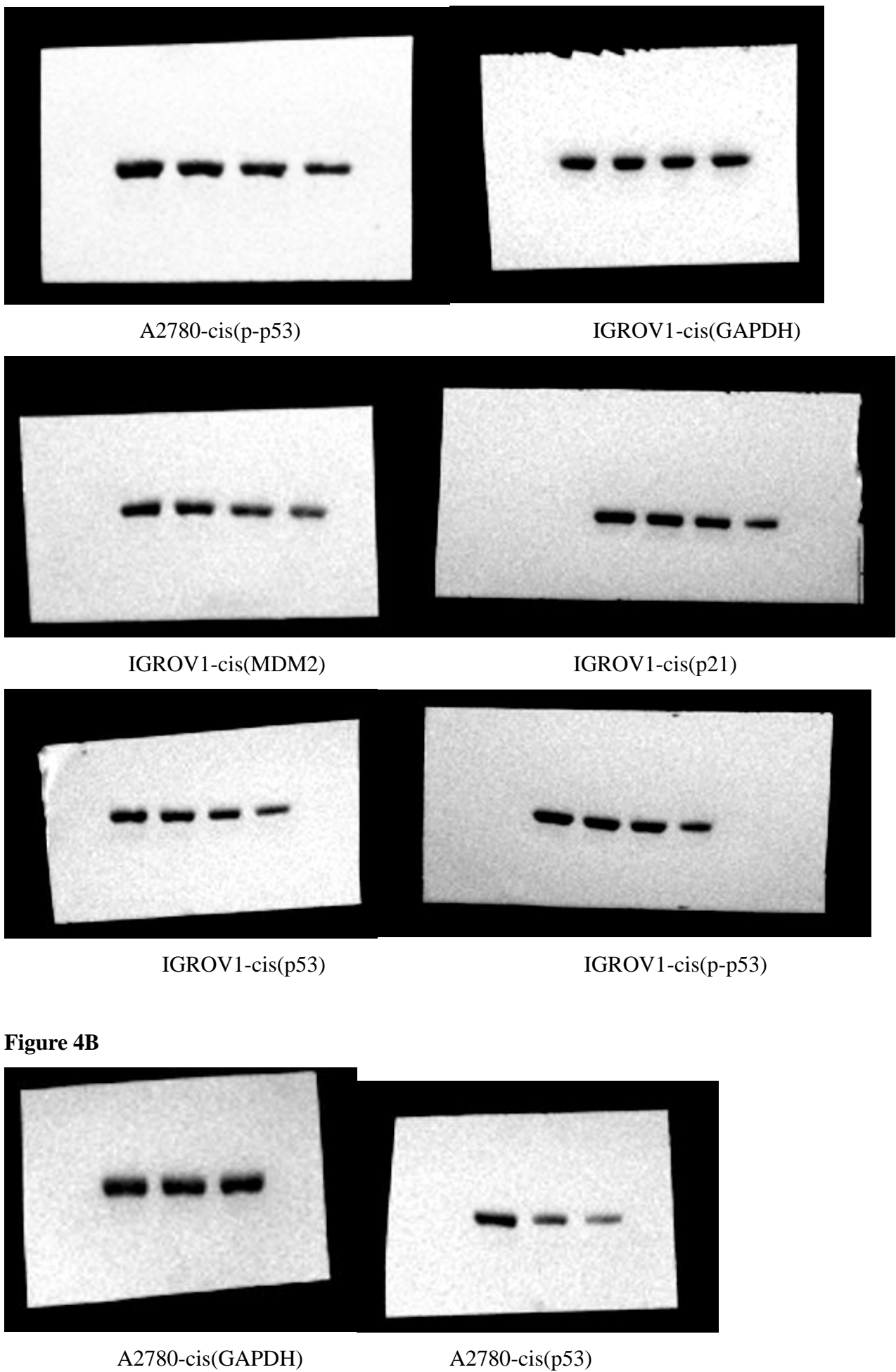
A2780-cis(p21)   A2780-cis(p53)
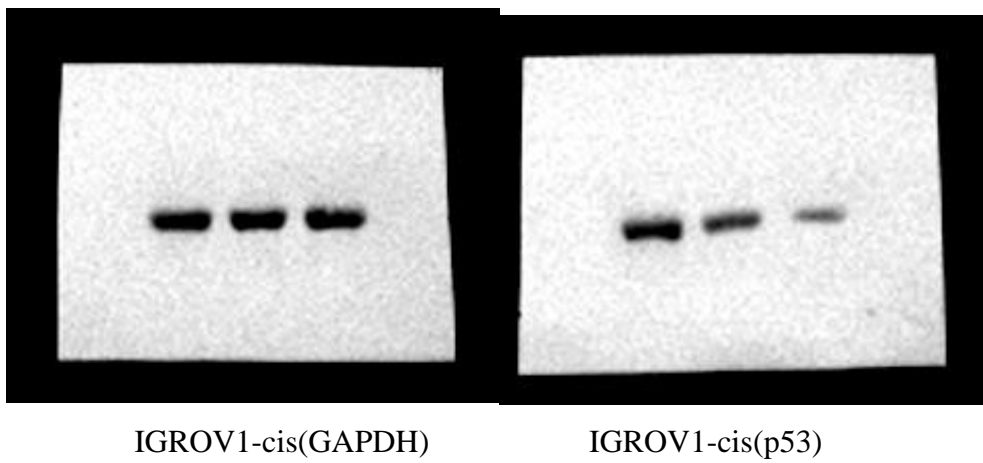
A2780-cis(p-p53)

IGROV1-cis(GAPDH)

IGROV1-cis(MDM2)

IGROV1-cis(p21)

IGROV1-cis(p53)

IGROV1-cis(p-p53)

Figure 4B

A2780-cis(GAPDH)

A2780-cis(p53)

IGROV1-cis(GAPDH)   IGROV1-cis(p53)

Figure 4E

GAPDH   MDM2

p21   p53

p-p53