TOPK inhibition accelerates oxidative stress-induced granulosa cell apoptosis via the p53/SIRT1 axis

JUNG-HWAN PARK[1], SANG-AH PARK[1], YOUNG-JU LEE[1], NA-RAE JOO[1], JONGDAE SHIN[2] and SANG-MUK OH[1,3]

Departments of [1]Biochemistry and [2]Cell Biology, College of Medicine, Konyang University; [3]Priority Research Center, Myunggok Medical Research Institute, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea

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Abstract. It has been suggested that oxidative stress involving reactive oxygen species (ROS) induces granulosa cell apoptosis, leading to follicular atresia, and that T-lymphokine-activated killer cell-originated protein kinase (TOPK) suppresses cancer cell apoptosis induced by several stimuli. However, it remains to be determined whether TOPK affects oxidative stress-induced granulosa cell apoptosis. The present study demonstrates that TOPK inhibition increases human granulosa COV434 cell apoptosis induced by hydrogen peroxide (H_2O_2). Co-treatment with the TOPK inhibitor, OTS514, in combination with H_2O_2 increased p53 acetylation and its expression, whereas it decreased Sirtuin 1 (SIRT1) expression, contributing to the promotion of apoptosis. In addition, the SIRT1 activator, resveratrol, or the SIRT1 inhibitor, Ex527, reduced or elevated H_2O_2-induced COV434 cell apoptosis, respectively. Furthermore, the p53 inhibitor, Pifithrin-μ, diminished the augmentation in poly(ADP-ribose) polymerase (PARP) cleavage induced by OTS514 plus H_2O_2, while the Mdm2 antagonist, Nutlin 3, increased PARP cleavage. Moreover, OTS514 further decreased the SIRT1 transcriptional activity decreased by H_2O_2, but promoted the H_2O_2-induced p53 or p21 transcriptional activity. Notably, the expression of exogenous p53 reduced SIRT1 transcriptional activity. Taken together, the findings of the present study demonstrate that TOPK inhibition promotes p53-mediated granulosa cell apoptosis through SIRT1 downregulation in response to H_2O_2. Therefore, it can be concluded that TOPK suppresses H_2O_2-induced apoptosis through the modulation of the p53/SIRT1 axis, suggesting a potential role of TOPK in the regulation of human granulosa cell apoptosis, leading to the promotion of abnormal follicular development.

Correspondence to: Dr Sang-Muk Oh, Department of Biochemistry, College of Medicine, Konyang University, 158 Gwanjeodong-ro, Seo-gu, Daejeon 35365, Republic of Korea
E-mail: sangmuk_oh@konyang.ac.kr

Abbreviations: TOPK, T-lymphokine-activated killer cell-originated protein kinase; SIRT1, sirtuin1; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; PARP, poly(ADP-ribose) polymerase; HDAC, histone deacetylase; MnSOD, manganese superoxide dismutase; Bax, Bcl-2-associated X protein

Key words: T-lymphokine-activated killer cell-originated protein kinase, apoptosis, granulosa cell, p53, Sirtuin 1

Introduction

The ovaries are known to be unique and crucial female organs which reproduce generations of eggs and secrete sex hormones for the female secondary sexual characteristics. However, the number of ovarian follicles secreted during a lifetime is limited. Follicles consist of several cells, including oocytes, granulosa cells and theca cells. The oocyte is surrounded by layers of granulosa cells and forms a direct connection with granulosa cells. It has been suggested that granulosa cells not only provide nutrient to oocytes, but also protect oocytes from oxidative stress during follicle development (1,2), and that reactive oxygen species (ROS)-mediated oxidative stress results in granulosa cell apoptosis to promote abnormal follicular development (3).

It has been reported that sirtuin 1 (SIRT1), a well-known NAD^+ dependent deacetylase (class III histone deacetylase), influences not only histone proteins, but also non-histone proteins, and regulates various biological processes involving aging, apoptosis or the stress response (4,5). In addition, SIRT1 is known to induce the phosphorylation of extracellular signal-regulated kinase (ERK) and inhibit the translocation of nuclear factor (NF)-κB into the nucleus, leading to suppression of granulosa cell apoptosis (6). It has been shown that various microRNAs (miRNAs or miRs) such as miRNA-590-3P or miRNA-494 suppress SIRT1 activity to promote apoptosis (7,8). It has been suggested that SIRT1 deacetylates lysine 382 of p53, thereby reducing p53 transcriptional activity and p53-mediated apoptosis, whereas p53 expression suppresses the transcription of SIRT1 gene induced by c-Myc (9-11).

T-LAK cell originated protein kinase (TOPK), a type of serine/threonine kinase, has been shown to be strongly expressed in various solid cancers, including colorectal cancer (12-14), lung cancer (15,16), gastric cancer (17), prostate cancer (18,19), ovarian carcinoma (20), nasopharyngeal

carcinoma (21) and esophageal squamous cell carcinoma (22). TOPK consists of 322 amino acids and has a functional similarity with MKK3/6, which activates p38 (23,24). However, it has been reported that TOPK phosphorylates ERK in EGF-stimulated HCT116 colorectal cancer cells or T47D cells (25,26) and phosphorylates JNK1 in ras-induced cell transformation or UVB-mediated signaling (27). In addition, TOPK has been shown to contribute to an increased metastasis of breast cancer cells by modulating the expression of MMP9 through the regulation of NF-κB activity in LPS signaling (28). On the other hand, it has been reported that TOPK directly binds to the DNA-binding domain of p53 and suppresses p53 transcriptional activity (29,30). However, whether TOPK affects oxidative stress-induced granulosa cell apoptosis remains to be determined.

In the present study, it was first revealed that TOPK inhibition increased p53-mediated granulosa cell apoptosis through SIRT1 downregulation upon H_2O_2 treatment. It was demonstrated that p53 negatively regulated SIRT1 transcriptional activity in response to H_2O_2, which was aggravated by TOPK inhibition. It was also found that TOPK inhibition promoted p53 stability, leading to an increase in H_2O_2-induced granulosa cell apoptosis. These findings provide evidence that TOPK suppresses H_2O_2-induced granulosa cell apoptosis through the regulation of the p53/SIRT1 axis.

Materials and methods

Cell culture and reagents. Human granulosa COV434 cells were purchased from Sigma-Aldrich; Merck KGaA. HeLa cells were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin and streptomycin (Thermo Fisher Scientific, Inc.). COV434 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin and streptomycin at 37˚C in a CO_2 incubator with 5% CO_2. Rabbit anti-TOPK (ab75987), rabbit anti-SIRT1 (ab32441), mouse anti-p53 (ab26), rabbit anti-phospho-p53 (Ser-15) (ab1431) and rabbit anti-acetyl p53 (K382) (ab75754) antibodies were purchased from Abcam. Rabbit anti-cleaved poly(ADP-ribose) polymerase (PARP) antibody (#9541) was purchased from Cell Signaling Technology, Inc. Mouse anti-β-actin antibody (A1975) was purchased from Sigma-Aldrich. OTS514, Z-VAD-FMK were purchased from Selleckchem; 2',7'-dichloroflurescin diacetate, Nutlin-3, Pifithrin-μ, resveratrol and Ex527 were purchased Sigma-Aldrich; Merck KGaA. Annexin V-FITC antibody and propidium iodide were purchased from Thermo Fisher Scientific, Inc. The luciferase assay kit was purchased from Promega Corporation.

Plasmids and transfection. The SIRT1 promoter linked luciferase reporter gene, SIRT1-Luc construct was generated. Briefly, an approximately 1 kb promoter region containing nucleotides -1 to -1,026 of the human SIRT1 gene was amplified using the Takara Ex Taq polymerase (Takara Bio, Inc.) and human genomic DNA (Promega Corporation) as a template. The amplified product was cloned into the *Nhe*I and *Xho*I sites of the pGL3-basic vector (cat. no. E1751, Promega Corporation). The specific primer sequences were as follows: Forward, 5'-CCGGCTAACCCATACTAGGCTTAAGG-3' and reverse, 5'-CCGCTCGAGCTTCCAACTGCCTCTCTG GC-3'. p53-GFP was a gift from Geoff Wahl (Addgene plasmid #11770). The pGL2-p53 promoter-Luc construct was a gift from Wafik El-Deiry (Addgene plasmid #16292) and the pGL2-p21 promoter-Luc plasmid was a gift from Martin Walsh (addgene plasmid #33021). The pcDNA3.1 or pEGFP-N1 vector was purchased from Invitrogen; Thermo Fisher Scientific, Inc. (cat. no. V79020) or Clontech (cat. no. 6085-1), respectively, and the V5-TOPK construct was previously described (31). Briefly, total RNA from HeLa cells was prepared, and cDNA synthesis and PCR were performed using Superscript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) and primers, 5'-CGCGGATCCCGATGGAAGGGATCAGTAATTT-3' harboring the *Bam*HI site (forward) and 5'-CGCTCGAGC GGGACATCTGTTTCCAGAGCTT-3' harboring the *Xho*I site (reverse). The PCR product digested with *Bam*HI/*Xho*I was inserted into the *Bam*HI/*Xho*I sites of pcDNA6/V5-His ABC (cat. no. V22020, Invitrogen; Thermo Fisher Scientific, Inc.), generating the V5-TOPK construct. Non-target siRNA and p53 siRNA were purchased from Dharmacon. COV434 cells growing in 12-well plates were transfected with 1 μg of pcDNA3.1 or V5-TOPK, 2 μg of pGL3-SIRT1 promoter-Luc, 1 μg of pEGFP-N1, pGL2-p53 promoter-Luc or pGL2-p21 promoter-Luc plasmids or 30 nM of non-target or p53 siRNA using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. At 24 h following transfection, cells were lysed, and luciferase assay was performed using cell lysate.

Flow cytometric analysis. A total of $1x10^6$ of COV434 cells growing on 60 mm dishes were pre-treated with inhibitors or activators, OTS514 (20 nM), Z-VAD-FMK (50 μM), resveratrol (20 μM), Ex527 (30 nM), Pifithrin-μ (1 μM), or Nutlin-3 (10 μM) for 2 h and H_2O_2 (0.1 mM) was then added for 24 h. Apoptosis analysis was performed using Annexin V-FITC and propidium iodide according to manufacturer's instructions (Thermo Fisher Scientific, Inc.), and data were acquired using a FACScalibur (BD Biosciences).

2',7'-Dichloroflurescin diacetate assay. COV434 cells growing on 60 mm dishes at 80% confluency were pre-treated with OTS514 (20 nM) for 2 h, and incubated at 37˚C with H_2O_2 (0.1 mM) for 24 h. The conditioned medium was removed, and fresh medium containing 10 μM H2DCF-DA was added. The cells were then incubated at 37˚C for 30 min, and then washed and trypsinized. Cells were resuspended using conditioned medium. DCF fluorescence was measured using a FACScalibur (BD Biosciences).

TUNEL assay. A total of $2x10^6$ of COV434 cells were seeded in 6-well plates and pre-treated with inhibitor or activator, OTS514 (20 nM), Z-VAD-FMK (50 μM), resveratrol (20 μM), Ex527 (30 nM), Pifithrin-μ (1 μM), or Nutlin-3 (10 μM), for 2 h, and then H_2O_2 (0.1 mM) was added for 24 h. Apoptosis was determined using the DeadEnd™ Fluorometric TUNEL System (G3250, Promega Corporation). The cells were fixed using 4% formaldehyde for 25 min at 4˚C, and then permeabilized by 0.2% Triton X-100 for 5 min at room temperature. The

cells were labeled using 50 μl of TdT reaction mix and incubated for 60 min at 37˚C and then data analysis was performed using a laser-scanning confocal microscope Zeiss LSM 710 (Zeiss AG).

Western blot analysis. Briefly, cells were harvested and lysed using lysis buffer containing 0.5% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4 and 40 mM NaCl. Briefly, 30 μg of cell lysate was separated on 8 or 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were blocked with 5% skim milk in Tris-buffered saline and Tween-20 (TBST) for 1 h and then incubated with anti-TOPK (1:5x10^6 in 5% Skim milk), p53 (1:1,000 in TBST), phospho-p53 (1:1,000 in TBST), acetyl-p53 (1:1,000 in 5% Skim milk), cleaved PARP (1:1,000 in TBST), SIRT1 (1:1,000 in TBST) and β-actin (1: 20,000 in 5% Skim milk) antibodies at 4˚C for overnight. The membranes were washed 3 times for 5 min using TBST, and then incubated at room temperature with goat anti-rabbi IgG, polyclonal (1:4,000 in 5% Skim milk, ADI-SAB-300-J, Enzo Life Sciences) or goat anti-Mouse polyclonal (1:4,000 in 5% Skim milk, ADI-SAB-100-J, Enzo Life Sciences) antibodies for 2 h. After washing, the blots were analyzed with SuperSignal West Pico chemiluminescent substrate (Pierce; Thermo Fisher Scientific, Inc.) and X-ray film. Each protein level was determined using Image J software (ver.1.52a; National Institute of Health).

Luciferase assay. A total of 1x10^5 of COV434 cells were seeded in 12-well plates. After 24 h, 1 μg of SIRT1, p53 or p21 promoter-luciferase reporter constructs plus 0.5 μg of the *pRL-SV40* gene were transfected into the COV434 cells using Lipofectamine 3000 reagent. At 24 h following transfection, the cells were pre-treated with OTS514 (20 nM), resveratrol (20 μM), Ex527 (30 nM), Pifithrin-μ (1 μM) or Nutlin-3 (10 μM) for 2 h, and then incubated at 37˚C with 0.1 mM H_2O_2 for 24 h. Cells were harvested and lysed. Firefly and *Renilla* luciferase activities were measured using Dual-Luciferase® Reporter assay system (E1910, Promega Corporation).

Protein stability assay. COV434 cells were seeded in 6-well plates. After 24 h, the cells were pre-treated with OTS514 (20 nM) for 2 h, and then treated with H_2O_2 (0.1 mM) for 24 h at 37˚C. 24 h after H_2O_2 treatment, cells were incubated with cycloheximide (10 μg/ml) (Sigma-Aldrich) for 0, 2, or 8 h, and then were harvested, lysed and subjected to western blot analysis.

Statistical analysis. Results are presented as the means ± standard deviation (SD) for at least 3 independent experiments in duplicate. Statistical analysis was carried out by one-way ANOVA with Tukey's test or two-way ANOVA followed by Bonferroni's correction. P-values <0.05 were considered to indicate statistically significant differences.

Results

TOPK inhibition increases H_2O_2-induced human COV434 granulosa cell apoptosis. Previous studies have demonstrated that oxidative stress induces granulosa cell apoptosis and SIRT1 downregulation mediated by oxidative stress contributes to this apoptosis (32-34). In addition, it has been suggested that TOPK functions as a survival effector in several cancer cells (35-37). Based on these findings, the present study wished to determine whether TOPK inhibition affects H_2O_2-induced granulosa cell apoptosis or SIRT1 expression. COV434 cells were treated with H_2O_2, OTS514 or H_2O_2 plus OTS514 for 24 h. Treatment of human COV434 granulosa cells with the TOPK inhibitor, OTS514, increased the number of TUNEL-positive cells induced by H_2O_2 stimulation (Fig. 1A), and significantly elevated the H_2O_2-induced cleavage of PARP (Fig. 1B). Of note, OTS514 treatment dose-dependently decreased TOPK expression upregulated by H_2O_2 and abolished the expression of SIRT1 downregulated by H_2O_2. Furthermore, the total p53 level, as well as its phosphorylation and acetylation were markedly increased upon H_2O_2 stimulation, and these effects were enhanced by OTS514 co-treatment. These data suggest that TOPK plays an important role in the regulation of p53 or SIRT1 expression in response to H_2O_2.

Subsequently, the effects of TOPK overexpression on the cleavage of PARP induced by H_2O_2 were examined. For this purpose, the COV434 cells were transfected with empty vector or TOPK overexpression vector for 24 h, and then incubated with a combination of H_2O_2 or OTS514 for 24 h. TOPK overexpression blocked the H_2O_2-induced cleavage of PARP (Fig. 1C). Furthermore, the effects of TOPK inhibition on endogenous ROS generation were investigated by 2',7'-dichlororoflurescin diacetate assay. The results revealed that targeting TOPK by OTS514 treatment enhanced the H_2O_2-induced ROS generation in granulosa cells, accelerating H_2O_2-induced the apoptosis (Fig. 1D).

Caspase inhibitor blocks granulosa cell apoptosis induced by co-treatment with H_2O_2 and OTS514. The present study then examined whether granulosa cell apoptosis induced by H_2O_2 and OTS514 is dependent on caspase-dependent apoptotic signaling pathways. COV434 cells were exposed to H_2O_2 only, H_2O_2 plus OTS514 or H_2O_2 plus OTS514 in combination with Z-VAD-FMK for 24 h. Co-treatment of the COV434 cells with OTS514 and H_2O_2 markedly increased the number of TUNEL-positive cells, compared to the cells stimulated with H_2O_2 only. However, co-treatment with the cell-permeable, irreversible pan-caspase inhibitor, Z-VAD-FMK, significantly decreased the number of TUNEL-positive cells (Fig. 2A). Consistent with this finding, flow cytometric analysis indicated that Z-VAD-FMK effectively diminished the number of Annexin V-positive cells induced by treatment with H_2O_2 and OTS514 (Fig. 2B). These findings suggest that caspase-dependent apoptosis pathways play a pivotal role in granulosa cell apoptosis induced by H_2O_2 and OTS514.

SIRT1 plays an important role in the regulation of H_2O_2-induced granulosa cell apoptosis. Subsequently, whether SIRT1 regulates H_2O_2-induced granulosa cell apoptosis was investigated. For this purpose, the SIRT1 activator, resveratrol, or the SIRT1 inhibitor, Ex527, were employed. The COV434 cells were incubated with H_2O_2 only, or H_2O_2 plus resveratrol or Ex527. As was expected, co-treatment with resveratrol decreased H_2O_2-induced apoptosis (Fig. 3A), whereas co-treatment with Ex527 increased apoptosis. In addition, western blot analysis revealed that co-treatment with resveratrol decreased

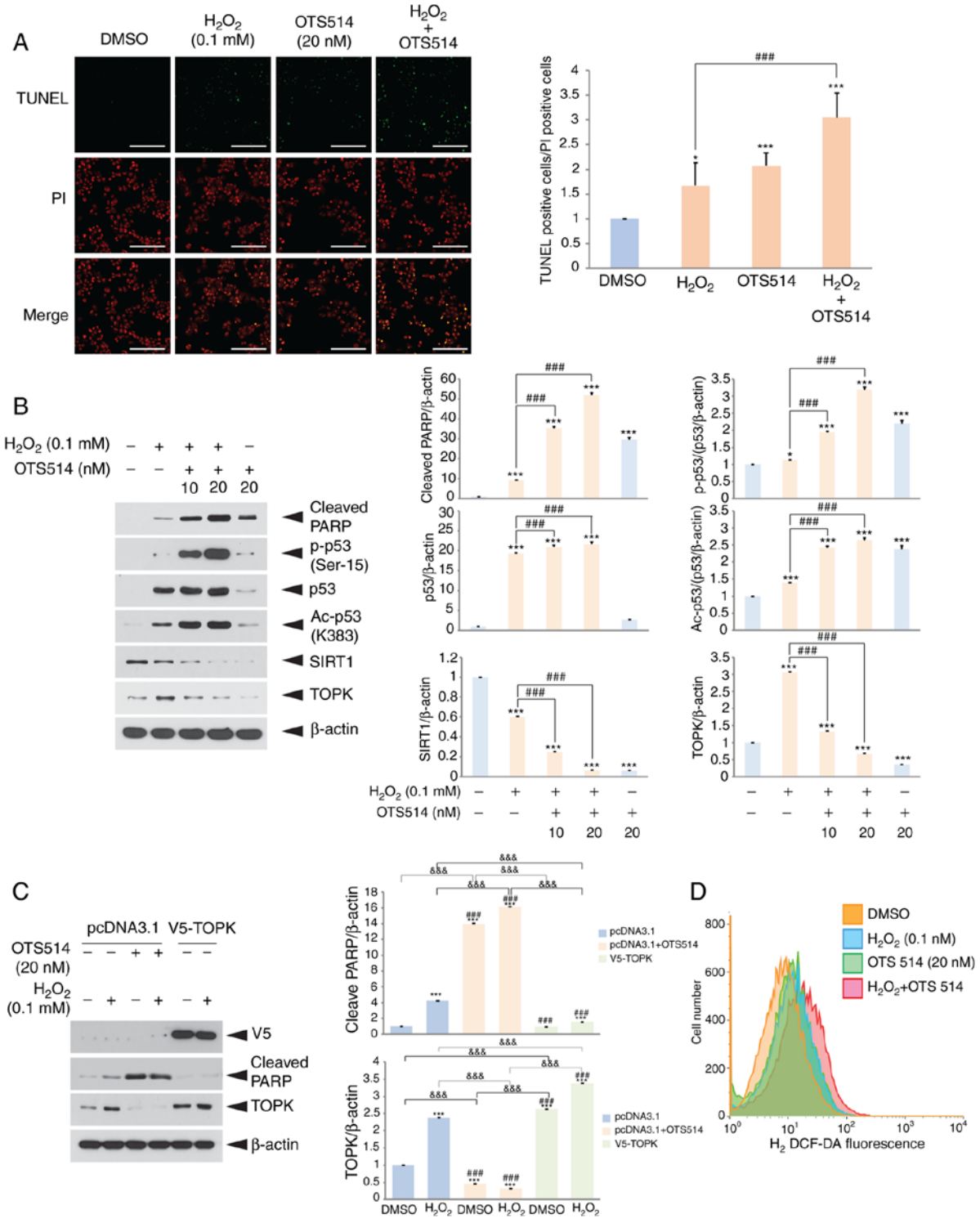

Figure 1. TOPK inhibition aggravates H_2O_2-induced human COV434 granuolsa cell apoptosis and decreases the endogenous SIRT1 level. (A) COV434 cells were treated with DMSO, H_2O_2, OTS514 or H_2O_2 plus OTS514 for 24 h, fixed and then examined for apoptosis by TUNEL assay. Cells were treated with OTS514 for 2 h prior to H_2O_2 exposure. Nuclei were stained with propidium iodide (PI). The ratio of TUNEL-positive cells to PI-positive cells is shown. Scale bar, 100 μm. (B) COV434 cells were treated with DMSO (-), H_2O_2 or H_2O_2 plus OTS514 for 24 h. Cell lysates were separated on SDS-PAGE, and then subjected to western blot analysis using indicated antibodies. The β-actin level was used as a loading control. (C) COV434 cells were transfected with empty vector, pcDNA3.1 or TOPK overexpression vector, V5-TOPK for 24 h, and then treated with a combination of H_2O_2 or OTS514 for 24 h. Western blot analysis was performed using indicated antibodies. (D) COV434 cells were incubated with DMSO, H_2O_2 or H_2O_2 in combination with OTS514 for 24 h. H2DCF-DA (10 μM) was added and incubated for 30 min. DCF fluorescence was measured by flow cytometry. Representative images of 3 independent experiments and graphs for quantitation are shown. *P<0.05, ***P<0.001 vs. DMSO. $^{\#\#\#}$P<0.001 vs. H_2O_2 or H_2O_2 control. $^{\&\&\&}$P<0.001 (two-way ANOVA with Bonferroni's correction).

the level of cleaved PARP level, as well as the acetylated p53 level induced by H_2O_2 (Fig. 3B); however, co-treatment with Ex527 increased the level of cleaved PARP and acetylated p53 level (Fig. 3C). These findings demonstrate that SIRT1 activity affects H_2O_2-induced granulosa cell apoptosis and they p53 level or activity is a key factor for the suppression of apoptosis.

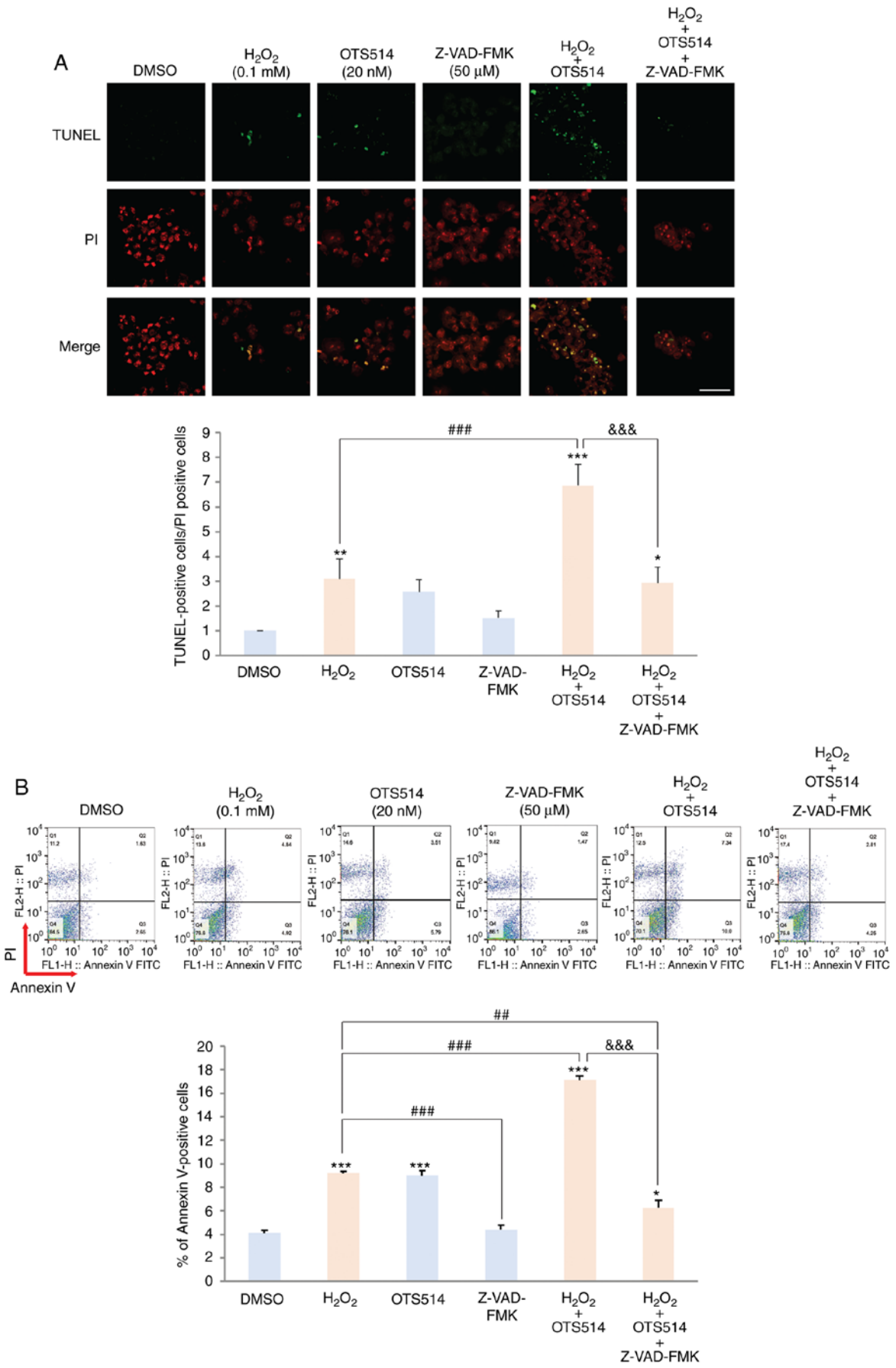

Figure 2. Caspase inhibition disrupts granulosa cell apoptosis induced by H_2O_2 and OTS514. COV434 cells were treated with DMSO, H_2O_2, OTS514 plus H_2O_2 or OTS514 plus H_2O_2 in combination with Z-VAD-FMK for 24 h, fixed, and then apoptosis was estimated by (A) TUNEL assay or (B) flow cytometry. Ratio of (A) TUNEL-positive cells to PI-positive cells or (B) % of Annexin V positive cells is shown. Scale bar, 50 μm. Representatives of 3 independent experiments are indicated. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. H_2O_2. $^{\&\&\&}$P<0.001 vs. H_2O_2 + OTS514.

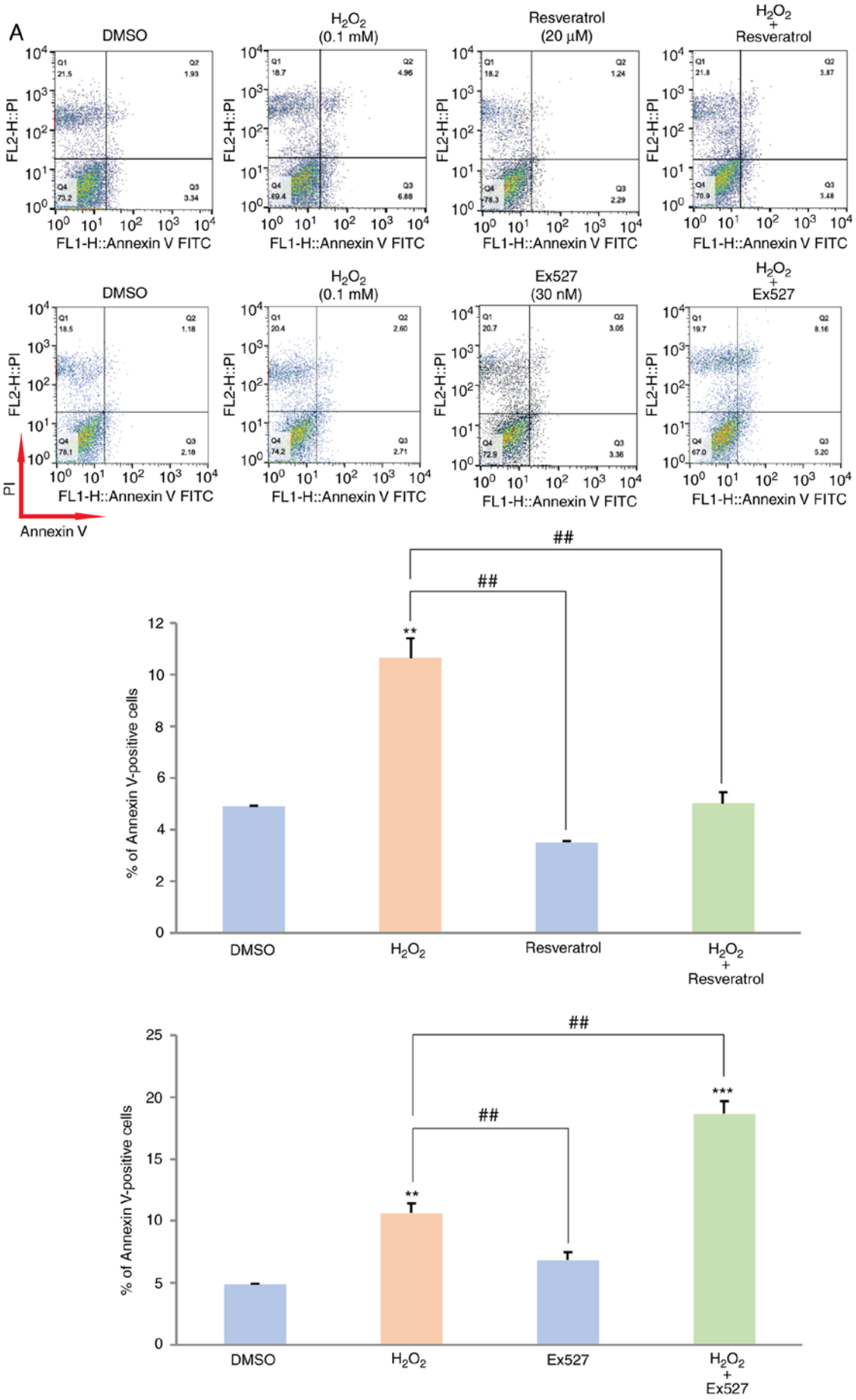

Figure 3. SIRT1 activity affects H_2O_2-induced granulosa cell apoptosis. (A) COV434 cells were incubated with a combination of H_2O_2 and resveratrol or Ex527 for 24 h. Apoptosis was analyzed by flow cytometry. The % of Annexin V-positive cells is indicated.

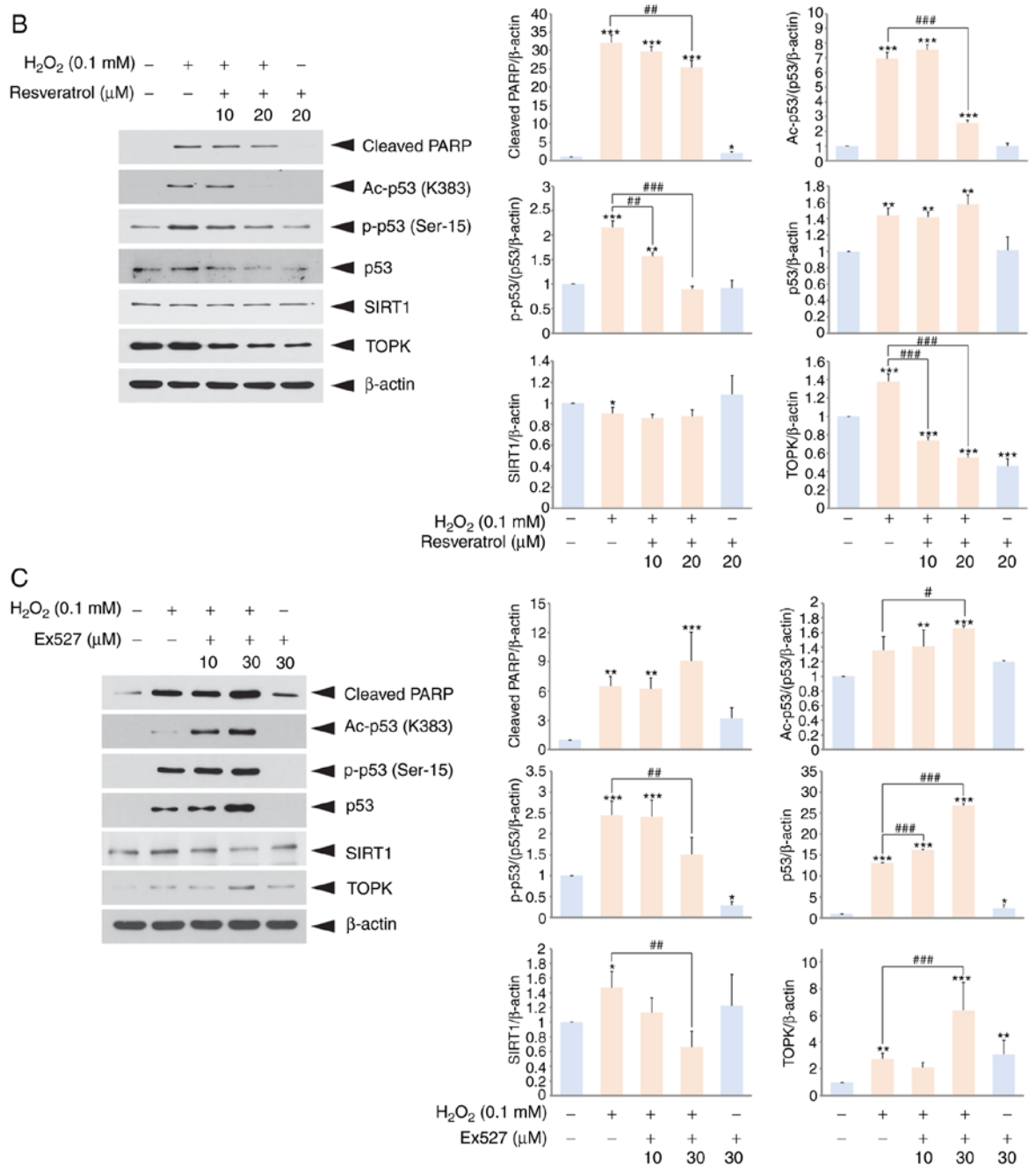

Figure 3. Continued. SIRT1 activity affects H_2O_2-induced granulosa cell apoptosis. (B and C) COV434 cells were treated with DMSO, H_2O_2 only, H_2O_2 plus Ex527 or resveratrol for 24 h. Cells were lysed and then subjected to western blot analysis using indicated antibodies. Graphs for quantification are indicated. Representatives of 3 independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. H_2O_2.

p53 inhibition suppresses H_2O_2 or OTS514-induced granulosa cell apoptosis. It has been suggested that tumor suppressor p53 is involved in granulosa cell apoptosis in response to oxidative stress or cAMP-mediated signaling (33,38). The present study then wished to determine whether p53 affects COV434 cell apoptosis upon H_2O_2 and OTS514 treatment. For this purpose, COV434 cells were treated with a combination of H_2O_2, OTS514 or the p53 inhibitor, Pifithrin-μ. The inhibition of p53 by Pifithrin-μ treatment decreased the H_2O_2-induced increase in the level of cleaved PARP (Fig. 4A). Co-treatment with OTS514 decreased TOPK expression, elevated the p53 level and decreased SIRT1 expression. In addition, TUNEL assay revealed that the inhibition of p53 decreased the H_2O_2-induced COV434 cell apoptosis by approximately 2-fold. However, co-treatment with OTS514 increased cell apoptosis (Fig. 4B). Taken together, these results demonstrate that p53 plays a key role in H_2O_2 or OTS514-induced granulosa cell apoptosis.

Inhibition of Mdm2 accelerates H_2O_2- and OTS514-induced granulosa cell apoptosis. To examine the effects of Mdm2 inhibition on H_2O_2 and OTS514-induced granulosa cell apoptosis, the Mdm2 antagonist, Nutlin 3, that inhibits the interaction of Mdm2 and p53 was employed. Treatment of the COV434 cells with Nutlin 3 elevated the levels of cleaved PARP, total p53, acetylated p53 or the phosphorylated p53 level, whereas it decreased the SIRT1 level upon H_2O_2 and OTS514

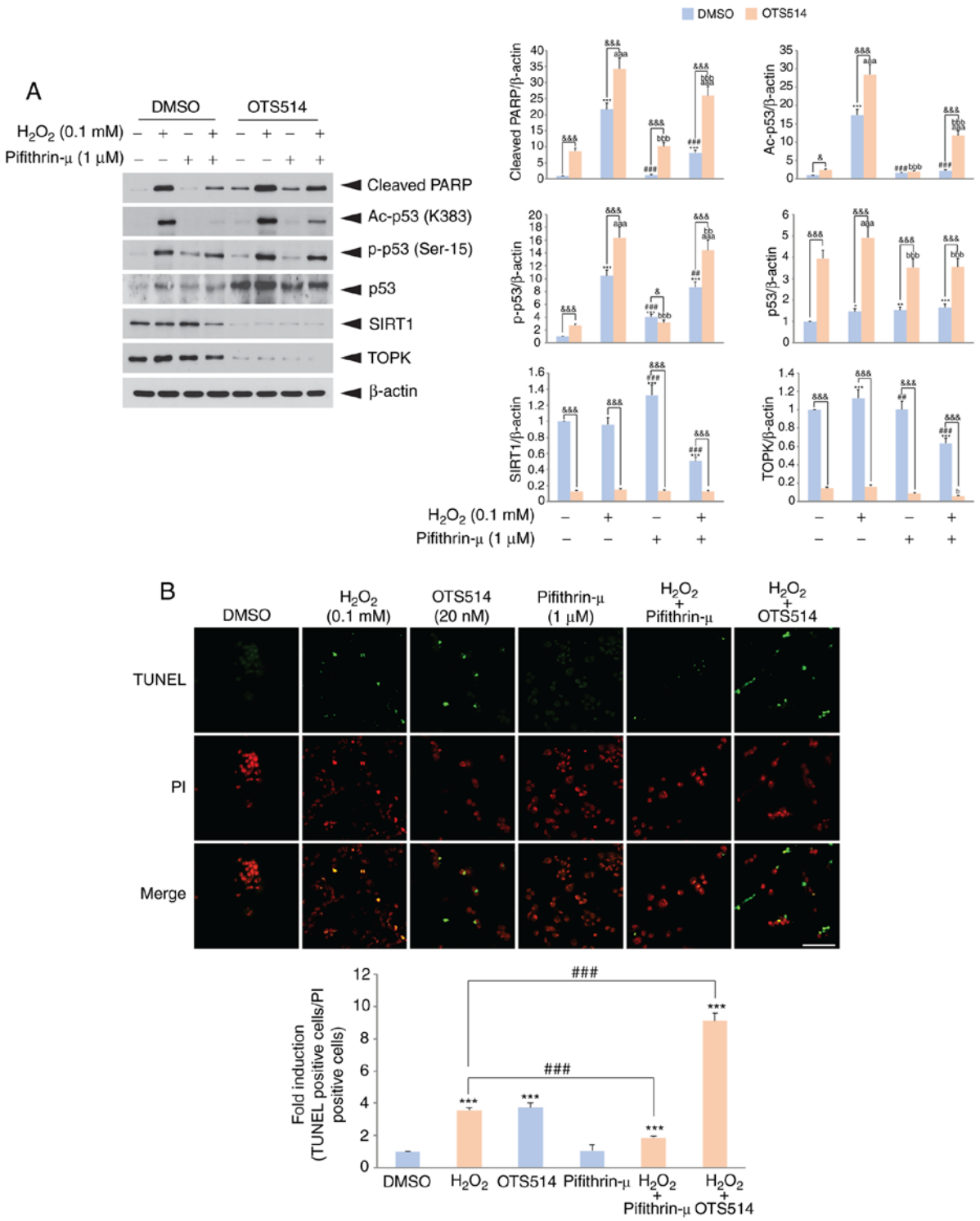

Figure 4. p53 inhibition alleviates granulosa cell apoptosis induced by H_2O_2 or OTS514. (A) COV434 cells were treated with a combination of H_2O_2 and OTS514 or Pifithrin-μ for 24 h. Cells were lysed and then subjected to SDS-PAGE and western blot analysis using indicated antibodies. Graphs for quantification are shown. (B) COV434 cells were incubated with a combination of H_2O_2 with Pifithrin-μ or OTS514 for 24 h. Cells were fixed, and apoptosis was analyzed by TUNEL assay. Ratio of TUNEL-positive cells to PI-positive cells is shown. Scale bar, 100 μm. Representatives of 3 independent experiments are indicated. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. H_2O_2. ^{aaa}P<0.001 vs. OTS514. ^{b}P<0.05, ^{bb}P<0.01, ^{bbb}P<0.001 vs. OTS514 + H_2O_2. $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 (two-way ANOVA with Bonferroni's correction).

treatment (Fig. 5A). Moreover, flow cytometric analysis indicated that the inhibition of the interaction of Mdm2 and p53 markedly increased the number of Annexin V-positive cells (Fig. 5B). Subsequently, whether p53 knockdown affects the H_2O_2-induced decrease in SIRT1 expression was examined. As was expected, the results revealed that p53 knockdown markedly restored SIRT1 expression to decrease the H_2O_2-induced cleavage of PARP (Fig. 5C). These findings suggest that both the level and activity of p53 may function as important factors in the regulation of granulosa cell apoptosis.

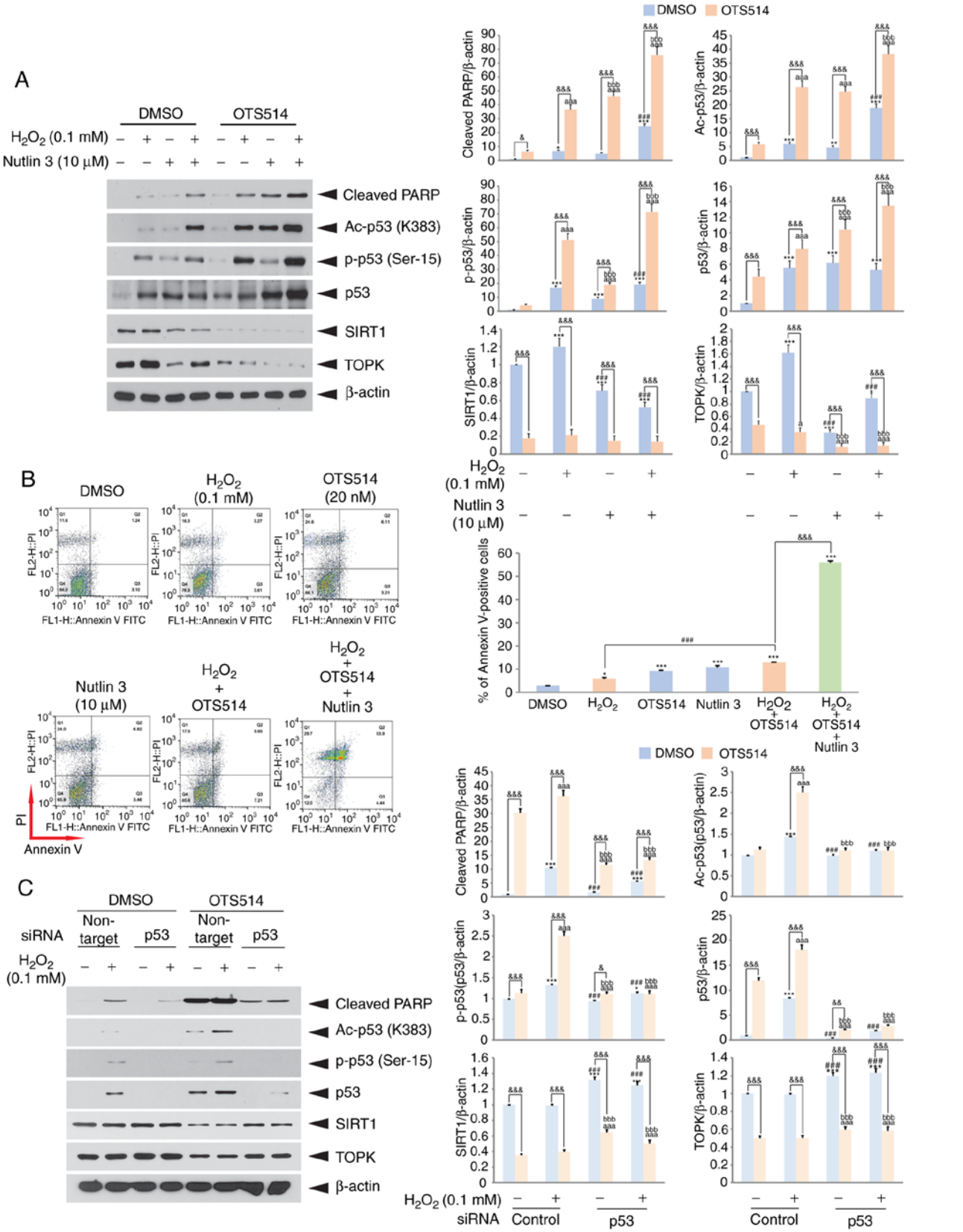

Figure 5. Mdm2 antagonist markedly accelerates the p53 level or granulosa cell apoptosis induced by H_2O_2 or OTS514. (A) COV434 cells were incubated in combination of H_2O_2 with OTS514 or nutlin 3 for 24 h. Cells lysates were subjected to western blot analysis using indicated antibodies. Graphs for quantitation are indicated. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. $^{\#\#\#}$P<0.001 vs. H_2O_2. ^{a}P<0.05, ^{aaa}P<0.001 vs. OTS514 control. ^{bbb}P<0.001 vs. H_2O_2 + OTS514. $^{\&\&\&}$P<0.001 (two-way ANOVA with Bonferroni's correction). (B) COV434 cells were treated with a combination of H_2O_2 with OTS514 or nutlin 3 for 24 h. Apoptosis was estimated using flow cytometry. The % of Annexin V-positive cells is shown. Representative images of 3 independent experiments are shown. *P<0.05, ***P<0.001 vs. DMSO. $^{\#\#\#}$P<0.001 vs. H_2O_2. $^{\&\&\&}$P<0.001 vs. H_2O_2+OTS514. (C) COV434 cells were transfected with non-target siRNA or p53 siRNA for 48 h, and then treated with a combination of H_2O_2 with OTS514 for 24 h. Western blot analysis was performed using indicated antibodies. Graphs for quantification are indicated. *P<0.05, ***P<0.001 vs. DMSO. $^{\#\#\#}$P<0.001 vs. H_2O_2. ^{aaa}P<0.001 vs. OTS514 control. ^{bbb}P<0.001 vs. H_2O_2 + OTS514. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 (two-way ANOVA with Bonferroni's correction).

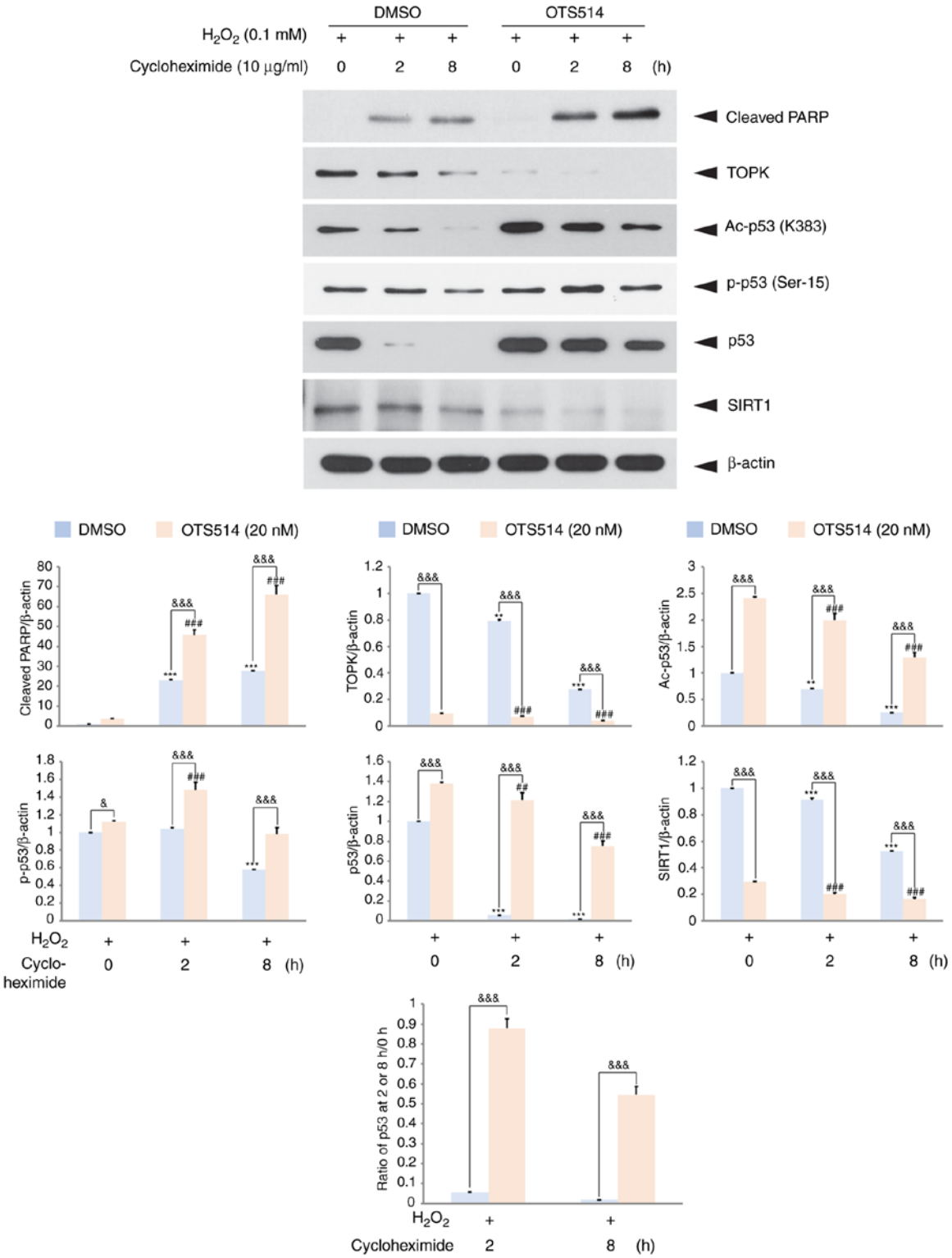

Figure 6. TOPK inhibition positively regulates p53 stability. COV434 cells were treated with OTS514 for 2 h prior to H_2O_2 treatment. At 24 h following H_2O_2 treatment, cells were incubated with cycloheximide for the indicated periods of time. Western blot analysis was performed using indicated antibodies. Graphs for quantification are shown. Representatives of 3 independent experiments are indicated. **P<0.01, ***P<0.001 vs. DMSO. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. H_2O_2. $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 (two-way ANOVA with Bonferroni's correction).

To examine whether p53 stability is affected by TOPK inhibition in H_2O_2-exposed granulosa cells, a cycloheximide chase experiment was then performed. The COV434 cells were treated with H_2O_2, OTS514 and cycloheximide, an inhibitor of protein biosynthesis. The level of acetylated p53, as well as the total p53 or cleaved PARP level in the H_2O_2- and OTS514-treated cells was elevated, while the TOPK or SIRT1 level was almost abolished 8 h following cycloheximide treatment compared with the cells stimulated with H_2O_2 only (Fig. 6). These findings suggest that TOPK inhibitor positively regulates p53 stability in H_2O_2-stimulated granulosa cells.

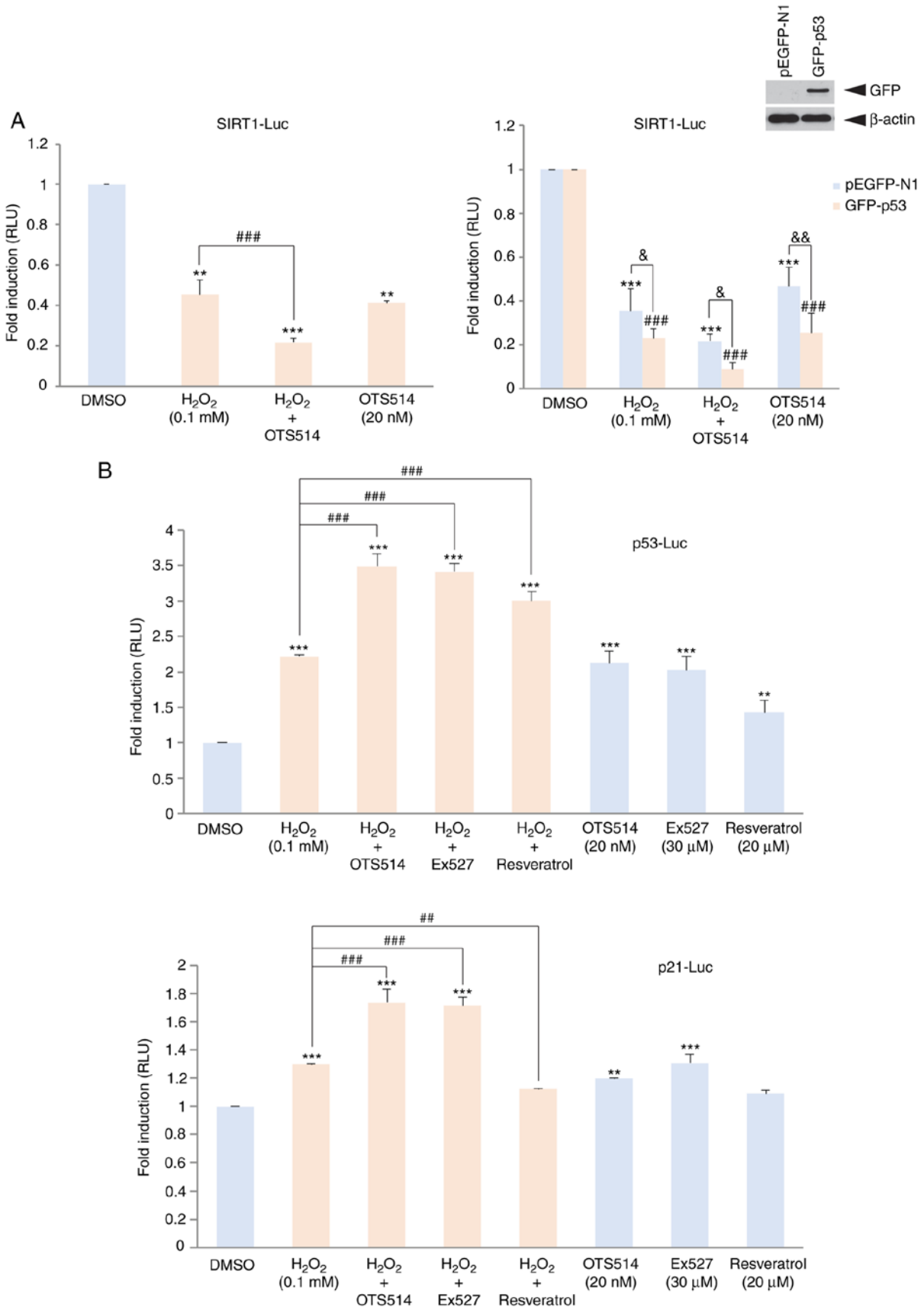

Figure 7. TOPK inhibition mitigates H_2O_2-downregulated SIRT1 transcriptional activity, but promotes p53 or p21 transcriptional activity upregulated by H_2O_2. (A) COV434 cells were transfected with SIRT1 promoter driven luciferase reporter construct (*SIRT1-LUC*) alone or SIRT1-LUC together with GFP-p53 or pEGFP-N1 plus *pRL-SV40* gene. At 24 h following transfection, cells were treated with DMSO, H_2O_2, OTS514 or H_2O_2 plus OTS514 for 24 h. (B) COV434 cells were transfected with p53-luciferase (*p53-LUC*) or p21-luciferase (*p21-LUC*) plus *pRL-SV40* gene. At 24 h post-transfection, cells were incubated in combination of H_2O_2 with OTS514, EX527 or resveratrol for 24 h. Firefly luciferase activity was estimated by normalization against *Renilla* luciferase activity. Relative luciferase unit (RLU) is indicated. **P<0.01, ***P<0.001 vs. DMSO. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. H2O2. $^{\&}$P<0.05, $^{\&\&}$P<0.01 (two-way ANOVA with Bonferroni's correction).

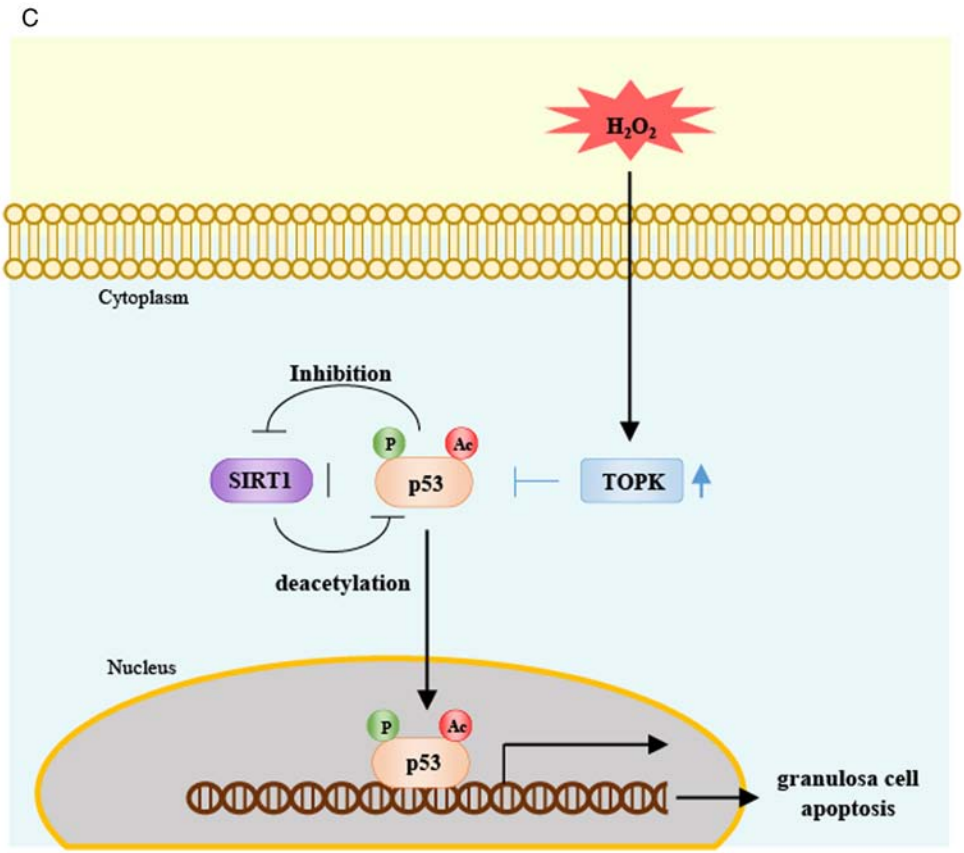

Figure 7. Continued. TOPK inhibition mitigates H_2O_2-downregulated SIRT1 transcriptional activity, but promotes p53 or p21 transcriptional activity upregulated by H_2O_2. (C) Schematic model for role of TOPK-p53-SIRT axis in regulation of granulosa cell apoptosis. TOPK is increased in response to H_2O_2, but TOPK downregulated by TOPK inhibitor OTS514 results in elevation of activity and expression of p53, which leads to the suppression of SIRT1 expression, thereby promoting p53-mediated granulosa cell apoptosis.

TOPK inhibition decreases the SIRT1 transcriptional level downregulated by H_2O_2, but increases the H_2O_2-induced p53 transcriptional level. It has been suggested that p53 negatively regulates SIRT1 expression. It has also been suggested that p53 negatively regulates SIRT1 expression (11). The present study thus examined the effect of TOPK inhibition on the SIRT1 or p53 transcriptional level, and the effect of p53 expression on the SIRT1 transcriptional level upon H_2O_2 stimulation. COV434 cells were transfected with SIRT1 or a p53 promoter-driven luciferase reporter construct. At 24 h following transfection, the cells were incubated with a combination of H_2O_2 or OTS514. The results indicated that stimulation of the COV434 cells with H_2O_2 resulted in a decrease in the SIRT1 transcriptional level and co-treatment with OTS514 promoted this decrease (Fig. 7A). On the other hand, co-treatment with OTS514 promoted the level of p53 or the p53 target, p21 transcriptional level induced by H_2O_2 (Fig. 7B). Notably, the expression of GFP-p53 diminished the SIRT1 transcriptional level downregulated by H_2O_2 (Fig. 7A). These findings demonstrate that upon H_2O_2 exposure, TOPK inhibition suppresses the SIRT1 transcriptional level and enhances the p53 transcriptional level, and that p53 negatively regulates SIRT1 expression. In addition, schematic model indicated that TOPK inhibition enhanced p53-mediated granulosa cell apoptosis through SIRT1 downregulation in response to H_2O_2 (Fig. 7C), suggesting the role of TOPK in the SIRT1/p53 regulatory axis.

Discussion

Granulosa cells are well known to be a class of cells that constitute a follicle for oocyte maturation. It has been proposed that granulosa cells and oocytes form gap junctions through connexins, which transfer low molecular weight molecules, such as cGMP, cAMP and calcium, and that granulosa cells transfer nutrients and regulatory factors for the growth and differentiation of oocytes (39-43). In addition, it seems that granulosa cells protect oocytes from oxidative stress involving ROS during follicle development. ROS have been reported to carry out functions that induce follicle rupture, and to regulate the expression of genes which induce oocyte maturation; however, accumulated ROS levels have been shown to cause oxidative stress that damages oocytes and granulosa cells (44-47). ROS mainly produced from the mitochondria cause mitochondrial damage to increase Bax expression, decrease Bcl-2 expression, and to release cytochrome *c* that forms the apoptosome, leading to the activation of the caspase cascade (48,49).

In this study, it was determined whether TOPK can affect oxidative stress-induced human granulosa cell apoptosis. It has been suggested that OTS514, a thieno[2,3-c]quinolone compound, strongly suppresses the growth of TOPK-positive cancer cells, including ovarian cancer, lung cancer or leukemia with low IC50 values ranging from 1.5 to 14 nM (50-52). OTS514 was shown to inhibit TOPK expression as well as TOPK kinase

activity. It has been suggested that OTS514 suppresses Forkhead box protein M1 (FOXM1) and FOXM1 transactivates the c-Myc promoter (52,53). Furthermore, c-Myc has been shown to positively regulate TOPK expression (54). In addition, FOXM1 enhances cyclin B1 that phosphorylates TOPK on the Thr-9 residue (55). Collectively, it seems that OTS514 inhibits TOPK activity and expression by suppressing FOXM1 or c-Myc. Based on these reports, an inhibitor was employed in the presents study to suppress TOPK expression or activity. The inhibition of TOPK activity or expression by the TOPK inhibitor, OTS514, exacerbated H_2O_2-induced COV434 granulosa cell apoptosis, which is dependent on caspase signaling pathways. It was also found that ROS production was markedly increased by co-treatment with H_2O_2 and TOPK inhibitor than with H_2O_2 only in COV434 granulosa cells. consistent with these findings, it has been previously demonstrated that TOPK inhibits the accumulation of H_2O_2 in RPMI7951 melanoma cells (56), induces the expression of manganese superoxide dismutase (MnSOD), an essential antioxidant enzyme localized in the mitochondria, in PC12 cells (57), and induces the expression of Nrf2, an antioxidant molecule, to reduce ROS (58). Collectively, the present study provides evidence that TOPK negatively regulates oxidative stress-induced granulosa cell apoptosis, thereby positively contributing to follicular development.

It has been suggested that oxidative stress induces tumor suppressor p53 to increase the expression of p53 target proteins, including p21, Bax and PUMA (9,59,60). In granulosa cells, oxidative stress has also been shown to result in PUMA expression, leading to the promotion of apoptosis through p53 induction (33). The findings of the presents study demonstrated that TOPK inhibition by OTS514 elevated the expression of total p53, acetylated p53 or the phosphorylated p53 level in granulosa cells. In addition, it was found that the inhibition of p53 degradation by Nutlin 3 accelerated H_2O_2-mediated granulosa cell apoptosis. It appears that the increase in the phosphorylated or acetylated p53 level, as well as the total p53 level contributed to the apoptosis. In agreement with these findings, it has been reported that TOPK binds to the p53 DNA binding domain, leading to the inhibition of expression of p53 and p53 target protein, p21, and thereby the induction of cell cycle arrest (29,30). Moreover, it has been proposed that the phosphorylation of p53 at the Ser-15 residue decreases its interaction with Mdm2, but increases its interaction with CBP/p300, thereby promoting p53 acetylation and activity (61,62). It is well known that p53-mediated apoptosis is due to the p53 translocation into the mitochondria. The present study confirmed that p53 inhibition by Pifithrin-μ, an inhibitor of p53 binding to the mitochondria alleviates granulosa cell apoptosis induced by H_2O_2 and OTS514. Previous studies have demonstrated that mitochondrial p53 accumulation promotes the opening of the mitochondrial permeability transition pore (MPTP), which is a protein channel for the regulation of mitochondrial membrane permeability (63-65). Mitochondrial p53 is known to induce Bak and Bax oligomerization and to suppress the anti-apoptotic activity of Bcl-xL and Bcl-2 (63,66,67).

On the other hand, it has been suggested that p53 deacetylation is mostly caused by HDACs, but SIRT1 specifically deacetylates p53 at the K382 residue (60,68,69). The present study found that the regulation of p53 acetylation by SIRT1 activator or inhibitor affected H_2O_2-induced granulosa cell apoptosis, and that H_2O_2-induced apoptosis was further decreased by resveratrol and Pifithrin-μ, and increased by Ex527 and Nutlin 3. Furthermore, TOPK inhibition diminished SIRT1 transcriptional activity downregulated by H_2O_2 treatment and p53 expression promoted the inhibitory effect of TOPK, which may be due to an increase in p53 expression induced by TOPK inhibition. In agreement with these findings, previous studies have suggested that p53 inhibits SIRT1 transcription by directly binding to the SIRT1 promoter, and that TOPK functions as a suppressor for p53 (11,30).

It has been suggested that SIRT1 binds to p53 and deacetylates the C-terminal p53 residue, Lys382, to negatively regulate the transcriptional activity of p53 (70,71). In the present study, the SIRT1 activator, resveratrol, or the SIRT1 inhibitor, Ex527, decreased or increased H_2O_2-induced TOPK expression, respectively. Although research on the regulation of the TOPK promoter remains elusive, p53 may transcriptionally modulate TOPK expression (16). Therefore, these findings may be due to the change in the H_2O_2 induction of SIRT1-mediated p53 transactivation, activity regulating TOPK expression. Taken together, these findings provide evidence for a novel role of TOPK in the p53/SIRT1 regulatory axis that mediates granulosa cell apoptosis. Therefore, TOPK may be a novel target molecule for the improvement of follicular development or oocyte maturation.

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Availability of materials and data

The data that support the findings of the present study are available from the corresponding author on reasonable request.

Authors' contributions

JHP and SAP conducted experiments, collected data, analyzed data and wrote the manuscript. YJL and NRJ performed experiments. JS provided some reagents and made suggestions during the performing of the experiments and data analysis. SMO designed and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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