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Toxoplasma gondii ROP18: potential to manipulate host cell mitochondrial apoptosis

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Abstract *Toxoplasma gondii* is an obligate intracellular parasite that may manipulate host cell mitochondrial apoptosis pathways. In our experiment, 293T cells were transfected with the p3×FLAG-CMV-Myc-ROP18 vector and expressed the ROP18-Myc fusion protein. Cell apoptosis was induced by 0.5 μg/mL actinomycin D (ActD) and was detected by Annexin V-FITC/PI assay. The cell mitochondrial membrane potential was determined by JC-1. Cytochrome *c* (Cyto-c) from mitochondria and the cytoplasm was measured by Western blot. The Bcl-2 and Bax coding gene expression levels were detected by real-time PCR. We found, in vitro, that *T. gondii* ROP18 significantly suppressed 293T cell apoptosis induced by ActD and maintained mitochondrial membrane potential and integrity, thereby preventing the release of Cyto-c from mitochondria into the cytoplasm. The ratio of Bcl-2/Bax in ROP18-overexpressing cells was significantly higher than that of the negative control. Therefore, we speculate that ROP18 could suppress host cell apoptosis via the mitochondrial apoptosis pathway in vitro.

Introduction

Toxoplasma gondii is an obligate intracellular parasite that can infect virtually any nucleated cells. Upon invasion, the parasite establishes itself within a specialized compartment, the parasitophorous vacuole (PV), which is circumscribed by the PV membrane (PVM). Several independent lines of evidence indicate that *Toxoplasma*-infected cells are resistant to induced apoptosis, and the type I and type III strains of *T. gondii* trigger host cell mitochondrial rearrangement in association with PVM (Sinai and Joiner 2001). After parasite invasion, a number of *T. gondii* proteins are secreted into the host cells (Laliberte and Carruthers 2008), such as rhoptry proteins, dense granules proteins, and microneme proteins, which are involved in parasite interactions with the host and play a key role in modulation of host cell replication and apoptosis (Ravindran and Boothroyd 2008).

Eukaryotes utilize apoptosis as one of their primary defense mechanisms against intracellular pathogens (Hasnain et al. 2003). Microbial infections trigger host mitochondria to instigate the intrinsic apoptotic pathway (Lamkanfi and Dixit 2010). It is known that *T. gondii* inhibits the release of cytochrome *c* (Cyto-c) from the intermembrane space of host cell mitochondria into the cytosol in response to a pro-apoptotic stimulus (Yang et al. 1997). However, the mechanism of distribution of host cell Cyto-c manipulated by *T. gondii* is not clear.

In our study, we overexpressed *T. gondii* ROP18 in 293T cells and evaluated the manipulation of cell apoptosis that was induced by actinomycin D (ActD) and determined the 293T cell's potential to modulate distribution of Cyto-c in mitochondria and cell cytoplasm, when overexpressing ROP18.

Liang Wu and Xiao Wang contributed equally to this work.

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The expressions of cell Bcl-2 and Bax were also specifically examined in our study.

Materials and methods

Construction of the overexpressing ROP18 vector

The plasmid construct used for in vitro expression was generated in p3×FLAG-Myc-CMV-24 (Sigma-Aldrich, St. Louis, MO, USA). The total DNA of *T. gondii* was purified using TissueGen DNA Kit (Generay Biotech Co., Ltd., China) as per manufacturer's instructions. The *ROP18* gene was amplified from *T. gondii* genomic DNA by PCR assay. The *ROP18* gene primers 5′-GGCAAGCTTATGTTTTCGGTAC AGCGGCCACCTCTTA-3′ (sense) and 5′-GGCGGATCCTTCTGTGTGGAGATGTTCCTGCTGTTC-3′ (antisense) yielded a 1662-nucleotide product specific to the *ROP18* coding gene (GeneBank no. CAJ27113.1). Amplification was carried out in a PCR mix containing 2 μL total DNA as a template, 8.5 μL ddH$_2$O, 12.5 μL PCR mix (Vazyme Biotech Co., Ltd, Nanjing, China), and 1 μL 10 μM of each primer (Generay Biotechnology Corporation, Shanghai, China). Thermocycling was performed at 95 °C for 1 min and 95 °C for 30 s; annealing was conducted at 58 °C for 20 s and extension at 72 °C for 90 s, for 25 cycles. The PCR product was digested with *Hin*dIII and *Bam*HI and ligated into the p3×FLAG-Myc-CMV-24 vector by using T4 DNA ligase (TaKaRa, Dalian, China) and was transformed into *Escherichia coli* DH5α strain. The right directional clone was confirmed by sequencing.

Cell culture, transfection, and *T. gondii* ROP18 detection

Human kidney 293T (293T) cells were cultivated in Dulbecco's modified Eagle's medium (HyClone, Beijing, China) supplemented with 15 % heat-inactivated fetal calf serum (Tianhang Biological Technology Co., Ltd, China), 100 U/mL penicillin, and 10 μg/mL streptomycin. All cells were cultured at 37 °C, in the presence of 5 % CO$_2$.

The 293T cells were transfected by Lipofectin reagent (RonBio Company, China). 0.5 μg plasmid DNA was diluted with 50 μL DMEM culture medium, and 2 μL Lipofectin reagent was diluted with 50 μL DMEM culture medium, followed by mixing of the two components in a 1.5-mL Eppendorf tube. For transfection, the final mixture was added into each well.

After transfection for 24, 36, and 48 h, the cells were harvested for Western blot analysis. They were boiled in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)-loading buffer (2 % (w/v) SDS, 62.5 mM Tris-HCl, and 10 mM sodium phosphate). The lysates were spun at 2000g, and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Merck Millipore Life Science, USA), and incubated with anti-myc tag antibodies (Vazyme Biotech Co., Ltd, Nanjing, China) and anti-actin antibodies (Abcam, Cambridge, MA, USA) diluted 1:5000 for 1 h. After washing, the strips were incubated with goat-mouse IgG conjugated to HRP (BOSTER, Wuhan, China) and analyzed via enhanced chemiluminescence (BOSTER, Wuhan, China).

Induction of cell apoptosis and detection of *T. gondii* ROP18

293T cells were cultured in six-well or 24-well dishes and divided into three groups including the transfection group, the negative control group, and the normal control group. In the transfection group, overexpressing ROP18 vector DNA was used, and the negative control group cells were transfected with empty vector DNA. The normal control group cells underwent addition of Lipofectin only. All cell apoptosis was induced by a concentration of 0.5 μg/mL ActD (Sigma-Aldrich, St. Louis, MO, USA), which was added into each plate well for 24 h before harvesting the cells. ROP18 expression in the transfection group was analyzed by Western blot.

Annexin V-FITC/PI analysis

In Annexin V-FITC/PI analysis, the 293T cells were cultured in 24-well dishes and divided into the transfection group, the negative control group, and the normal control group. The experiment started when vector DNA and Lipofectin were added in transfection group and negative control group. The normal control group only underwent addition of Lipofectin. All three groups' cell apoptosis was induced by ActD for 24 h prior to harvesting. After transfection for 24, 36, and 48 h, the 293T cells were collected and stained with Annexin V-FITC through the Annexin V-FITC/PI kit (Vazyme, Nanjing, China), and apoptotic cells were identified and quantified by flow cytometry (FACSCalibur, BD Company, USA). Briefly, 293T cells (1×10^6/well) were washed with PBS and incubated with 1× binding buffer, propidium iodide (PI), and Annexin V-FITC for 10 min at room temperature. The apoptotic cells were analyzed on a fluorescence-activated cell sorter (FACSCalibur, BD Company, USA). This assay was repeated six times.

JC-1 analysis

The 293T cells were cultured in 24-well dishes and divided into the transfection group, the negative control group, and the normal control group. This assay was repeated six times. ActD induced cell apoptosis for 24 h prior to harvesting, and JC-1 assay kits (Beyotime, Nantong, China) were used to assess the mitochondrial status of 293T cells. After transfection for 24, 36, and 48 h, 293T cells were washed with PBS and diluted with a dilution buffer to 1×10^6 cell/mL, and

aliquots of 500 μL were transferred to Eppendorf tubes, followed by the addition of 0.5 μL of JC-1 stock solution (3 mM JC-1 in DMSO). All the tubes were kept in a water bath at 37 °C for 20 min. All the samples were analyzed by flow cytometry. The mean intensity of red fluorescence and green fluorescence was recorded, and the ratio of red fluorescence intensity and green fluorescence intensity was considered as the cell mitochondrial membrane potential.

Western blot analysis of Cyto-c distribution

The 293T cells were cultured in six-well dishes and divided into the transfection group, the negative control group, and the normal control group. Cell apoptosis was induced by ActD for 24 h prior to harvesting. The experiment was stopped for distribution analysis of Cyto-c in 293T cells by Western blotting, after transfection for 24, 36, and 48 h, respectively. COXIV was used as cell mitochondrial protein reference, and actin was utilized as cell cytoplasm protein reference. Cell proteins were separated by a mitochondrial protein and cytoplasm protein isolation kit (Beyotime, Nantong, China) and were boiled in SDS-PAGE-loading buffer. The lysate proteins were separated by SDS-PAGE; transferred to nitrocellulose membranes and incubated for 1 h with anti-Cyto-c, COXIV, and anti-actin antibodies (Abcam, Cambridge, MA, USA); and diluted 1:5000. After washing, the strips were incubated with goat-rabbit IgG conjugated to HRP and analyzed through enhanced chemiluminescence. This assay was repeated six times.

Real-time PCR analysis

The 293T cells were cultured in six-well dishes and divided into the transfection group, the negative control group, and the normal control group. ActD induced cell apoptosis for 24 h prior to harvesting. After transfection for 24, 36, and 48 h, the 293T cells were harvested for reverse transcription real-time PCR analysis. Total cellular RNA was purified by RNA Isolater Total RNA Extraction Reagent (Vazyme, Nanjing, China). The real-time PCR assay was carried out per a former study (Wu et al. 2009), using the Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc. Berkeley, CA, Hercules, California, USA) and AceQ®qPCR SYBR® Green Master Mix (Vazyme, Nanjing, China). Each assay was repeated six times. Briefly, the optimized real-time PCR reaction contained 1 μL 293T cell cDNA, 0.5 μL of 10 μM each primer, 12.5 μL real-time PCR mix, and 10.5 μL ddH$_2$O to a final reaction volume of 25 μL. The *Bax* gene primers were as follows: 5′-TCCACCAAGAAGCTGAGCGAG-3′ (sense) and 5′-GTCCAGCCCATGATGGTTCT-3′ (antisense). The *Bcl-2* gene primers were as follows: 5′-TCCACCAAGA AGCTGAGCGAG-3′ (sense) and 5′-GTCCAGCC CATGATGGTTCT-3′ (antisense). The standard PCR started with initial denaturation at 95 °C for 30 s, followed by

35 cycles of amplification at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s. The amplicons specific to *Bcl-2* gene and *Bax* gene were cloned into the pTG19-T vector (GENEray, Shanghai, China) and transformed into DH5α. To calculate the PCR efficiency, these plasmids provided standard curves for the relative quantification of *Bcl-2* and *Bax* genes. The two plasmids with known concentrations were utilized in a 10-fold dilution series (10^6 copies/μL–10^1 copies/μL) for standard curve establishment.

Statistical analysis

All values were expressed as the mean ± standard error (SE). Statistical analysis was performed by ANOVA followed by Fisher's protected least significant differences test through Stata 7.0 software (Stata Corporation, College Station, TX, USA). A *P* value <0.05 was considered statistically significant.

Results

ROP18 expressed in 293T cell

T. gondii ROP18 gene was completely inserted into p3×FLAG-Myc-CMV-24 vector (Fig. 1a). *T. gondii* ROP18 fused with myc tag was detected in 293T cells by Western blot after transfection for 24, 36, and 48 h, and the expression was increased when the time was prolonged. After transfection for 36 and 48 h, the ROP18-myc fusion protein expression in 293T cells was obvious (Fig. 1b).

T. gondii ROP18 regulate 293T cell apoptosis

When treated with 0.5 μg/mL ActD, ROP18 expression could be detected in 293T cells in all groups (Fig. 2). The 293T cell apoptosis rates in the transfection group were lower than those in the negative control group and the normal control group after transfection for 24, 36, and 48 h. In the 48-h treatment group, the apoptosis rate of the transfection group was significantly lower than that of the negative control group and the normal control group (*P* < 0.05) (Fig. 3).

T. gondii ROP18 regulates 293T cell mitochondrial membrane potential

After transfection for 24 and 36 h, the ratio of red fluorescence intensity to green fluorescence intensity for 293T cells in the transfection group was much higher than that of the negative control group and the normal control group (*P* < 0.05). However, at 48 h, there was no significant difference between groups in the ratio of red fluorescence to green fluorescence (*P* > 0.05) (Fig. 4).
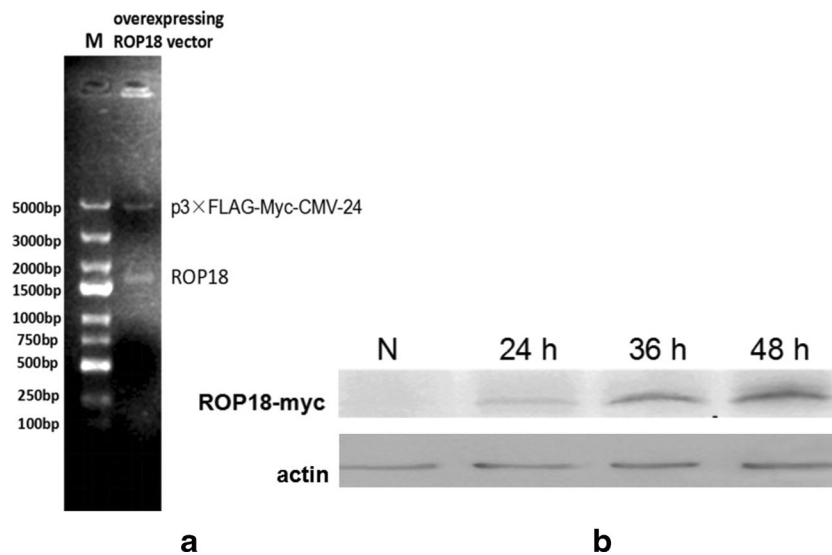
a

b

Fig. 1 Overexpressing *T. gondii* ROP18 vector construction and expression in 293T cells. **a** Overexpressing ROP18 vector analysis by restriction enzyme digestion. *M* DNA marker. **b** Detection overexpressing *T. gondii* ROP18 by Western blotting assay. The overexpressing ROP18-myc fusion was detected when transfected in 24, 36, and 48 h. The actin of 293T cell was used as the reference. The overexpressing ROP18 was obviously detected in 36 and 48 h. *N* the 293T cells transfected with empty vector in 48 h as the negative control

Cyto-c distribution in the mitochondria and cytoplasm

In 293T cell mitochondria, there was much greater Cyto-c in the transfection group than in the negative control group and in the normal control group. At 24 and 36 h post transfection, the levels of Cyto-c were not significantly different between the three groups. When transfection time was prolonged to 48 h, the levels of Cyto-c were dramatically decreased in the negative control group and the normal control group but not in the transfection group. At 48 h, the Cyto-c levels in the transfection group were much greater than those in the negative control group and the normal control group ($P < 0.05$) (Fig. 5a).

In 293T cell cytoplasm, the Cyto-c level in the transfection group was much lower than that in the negative control group and the normal control group when the transfection continued for 24 h ($P < 0.05$). When the transfection time was extended to 36 h, the transfection group's Cyto-c level was much lower than that in the negative control group ($P < 0.05$). However, at 48 h, despite dramatic increases of Cyto-c in all three groups, there was no significant difference between groups ($P > 0.05$) (Fig. 5b).

Bcl-2/Bax coding gene expression

The ratios of Bcl-2/Bax coding genes were decreased after transfection. When transfected for 24 h, the ratio of Bcl-2/Bax coding genes indicated no significant differences among the transfection group, the negative control group, and the normal control group ($P > 0.05$). On the other hand, when the time was prolonged to 36 h, the ratio of Bcl-2/Bax coding gene in the transfection group was much higher than that in the negative control group ($P < 0.05$). When the time was prolonged to 48 h, the ratio of Bcl-2/Bax coding genes in the transfection group was much

higher than that in the negative control group and the normal control group ($P < 0.05$) (Fig. 6).

Discussion

In our study, we established that *T. gondii* ROP18 had the ability to inhibit 293T cell apoptosis *in vitro*, which was induced by 0.5 μg/mL ActD. Meanwhile, we discovered that ROP18 maintained the host cell mitochondrial membrane potential, the balance of Bcl-2/Bax expression, and prevented cytochrome *c* leakage. We speculate that *T. gondii* ROP18 has the potential to maintain host cell mitochondrial membrane integrity and manipulate host cell mitochondrial apoptosis.

T. gondii is an obligate intracellular parasite and was capable of inhibiting apoptosis in the pathogenesis of both acute and chronic infections (Carmen et al. 2006). The capability to inhibit cell apoptosis is very important for the parasite because it provides a stable environment for parasitic proliferation. A
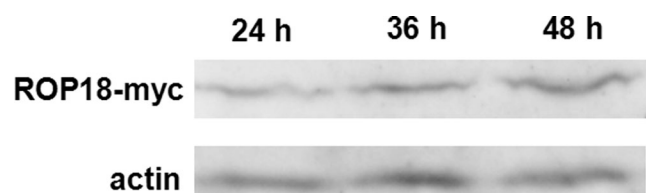

Fig. 2 The 293T cells overexpressing *T. gondii* ROP18 when treated with 0.5 μg/mL ActD was detected by Western blotting assay. The 293T cells was transfected with plasmid DNA for 24, 36, and 48 h and treated with ActD for 24 h before harvesting the cells. The result indicated that ActD treatment had no significant influence on ROP18 overexpressing
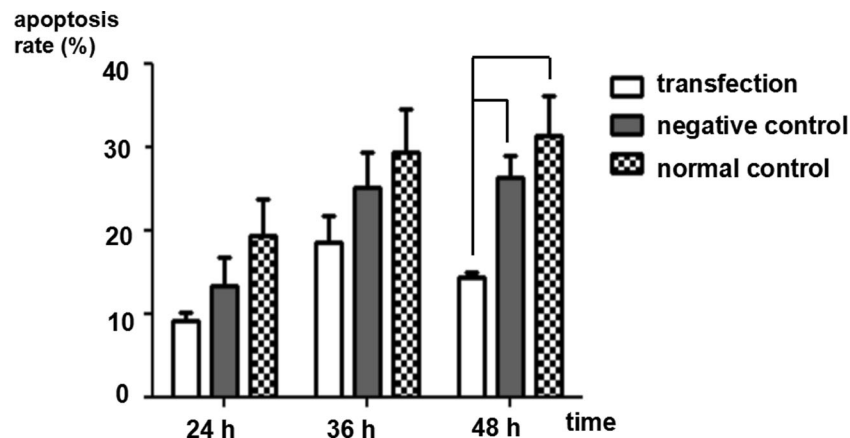
Fig. 3 Overexpressing ROP18 modification 293T apoptosis induced by 0.5 μg/mL ActD. The apoptosis cells were consisted of both Annexin V-single positive and Annexin V/PI-double positive. In the negative control group and the normal control group, the apoptosis rate was higher than that of the transfection group in 24 and 36 h, but not significant ($P > 0.05$). In 48 h, the apoptosis rates in the negative control and the normal control group were significantly higher than the transfection group ($P < 0.05$)

large volume of literature has reported that *T. gondii* had the ability to modify host cell apoptosis by inhibiting the release of host cell cytochrome *c*, which is a pro-apoptotic stimulus (Hippe et al. 2009). Finding the key inhibitors of apoptosis by *T. gondii* is crucial in the study of its pathogenesis. In this study, we overexpressed *T. gondii* ROP18 in 293T cells. Using an overexpression system, we could study ROP18 function accurately without interference from other *T. gondii* proteins.

ROP18 is a serine/threonine kinase secreted from rhoptry organelles. As a member of the ROP2 family, it is located in the rhoptries and is secreted to the PVM and host cell cytoplasm during parasite invasion (Fentress and Sibley 2011). In this location, through phosphorylation, ROP18 can modify and/or control other PVM proteins (such as another rhoptry protein or a dense granule protein) and host cell functions. There are two related events which have already been described, namely the phosphorylation of ROP4 on several serine/threonine residues after translocation in the PVM (Carey et al. 2004), and the phosphorylation of host IκB that correlates with the activation of NF-κB, which is required for the inhibition of apoptosis (Molestina and Sinai 2005). A study by Sinai et al. (Sinai and Joiner 2001) found that *T. gondii* had the ability to mediate host cell mitochondrial association with PVM. We propose that the intimate contact between host cell mitochondria and PVM might provide a better channel for the interaction of parasite proteins and host mitochondria.

Serine/threonine kinases in mammalian cells are also important for the modulation of cell apoptosis. Kennedy et al.
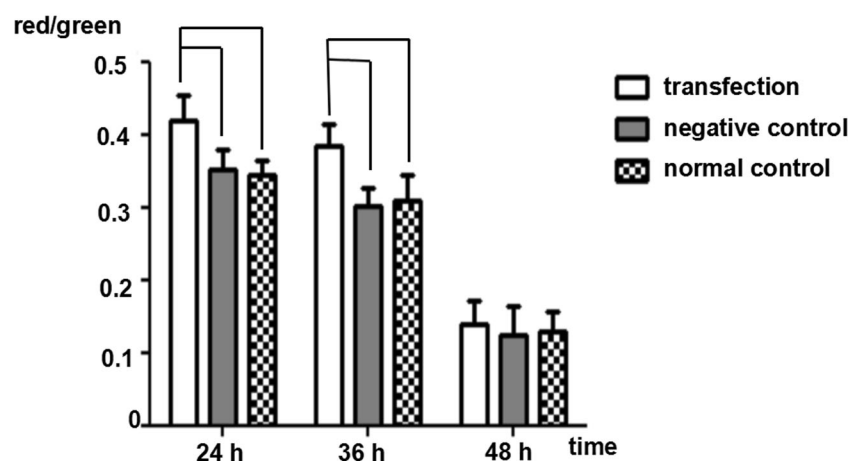

Fig. 4 Overexpressing *T. gondii* ROP18 manipulating 293T cell mitochondrial membrane potential. The ratio of red fluorescence intensity and green fluorescence intensity was considered as the cell mitochondrial membrane potential. In 24 and 36 h, the cell mitochondrial membrane potential in the transfection group was significantly higher than the negative control group and the normal control group ($P < 0.05$). In 48 h, there was no significant difference in the transfection group, the negative control group, and the normal control group ($P > 0.05$)
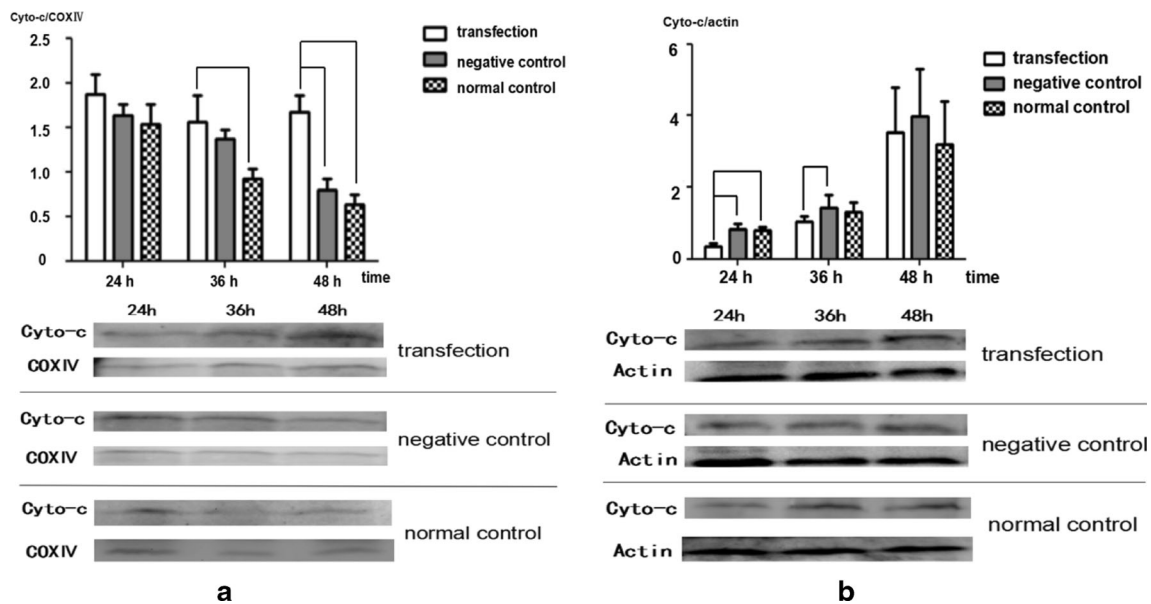
Fig. 5 Overexpressing *T. gondii* ROP18 manipulating 293T cell Cyto-*c* distribution. **a** Cyto-*c* distribution in 293T cell mitochondria. COXIV was used as the reference for mitochondria protein. In 24 and 36 h, the Cyto-c expression in mitochondria in the transfection group is higher than that in the negative control group and the normal control group, but not significant ($P > 0.05$). In 48 h, there were significant differences in the transfection group compared to that in the negative control group and the normal control group ($P < 0.05$). **b** Cyto-*c* distribution in 293T cell cytoplasm. Actin was used as the reference for cytoplasm protein. In 24 h, the Cyto-*c* expression in cytoplasm in transfection group is significantly lower than that in the negative control group and the normal control group ($P < 0.05$). In 36 h, the Cyto-*c* expression in cytoplasm in transfection group is significantly lower than that in the negative control group ($P < 0.05$). In 48 h, there was no significant difference in the transfection group, the negative control group, and the normal control group ($P > 0.05$)

(1999) discovered that serine/threonine kinases in human cells inhibited cell death by preventing the release of cytochrome *c* from mitochondria. Cross et al. (2000) focused on the role of mammalian serine/threonine kinases and revealed that this kinase family plays a role in apoptosis via interference with the activity of the mitogen-activated protein kinase (MAPK) family, specifically p42/44 ERK, p38 MAPK, c-Jun N-terminal kinase (JNK), cyclic AMP-dependent protein kinase (PKA), protein kinase B (PKB), or Akt and protein kinase C (PKC). Because of the serine/threonine kinase activity of

T. gondii ROP18, we hypothesized that *T. gondii* ROP18 maintained host mitochondrial membrane integrity and inhibited cytochrome *c* release into cytoplasm.

ActD is a widely used inducer of apoptosis in a variety of cells in vitro and in vivo. It binds to cell's DNA and inhibits RNA and protein synthesis (Fujita et al. 1996) and exerts no inhibition of ROP18 serine/threonine kinases activities. In our results, the expression of ROP18 was detected at 24, 36, and 48 h, and the apoptosis rate in overexpressing cells had decreased moderately in 24 and 36 h, but had decreased significantly in 48 h. The
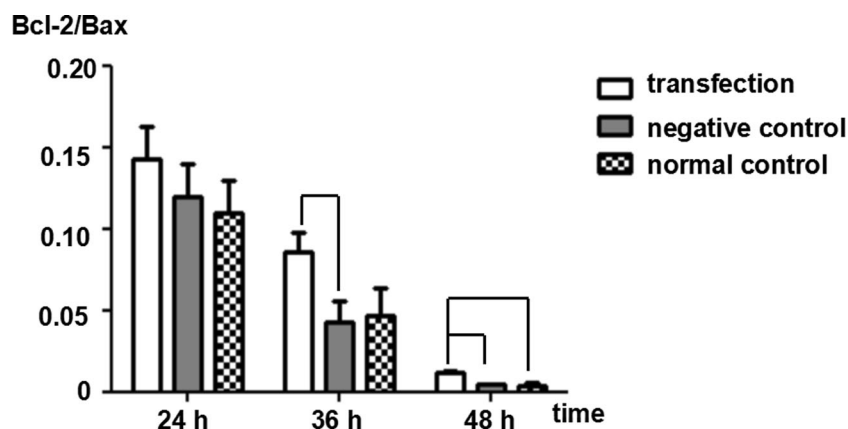

Fig. 6 Overexpressing *T. gondii* ROP18 manipulating 293T cell Bcl-2 and Bax coding gene expression. In 24 h, the Bcl-2/Bax value had no significant difference in the transfection group, the negative control group, and the normal control group ($P > 0.05$). In 36 h, the Bcl-2/Bax value of transfection group was significantly higher than that in negative control group ($P < 0.05$). In 48 h, the Bcl-2/Bax value of transfection group was much higher than that in the negative control group and the normal control group ($P < 0.05$)

reason for delayed apoptosis is that ROP18 has only a moderate ability to manipulate host cell apoptosis. The dramatic activity of downregulating host apoptosis needs some time to be detected by Annexin V-FITC/PI assay.

Release of cytochrome c from mitochondria triggers the activation of cell apoptosis (Goldstein et al. 2000). However, the intact mitochondrial membrane could block the release of cytochrome c from mitochondria and suppress cell apoptosis (Li et al. 2000). In our study, we determined mitochondrial membrane potential and cytochrome c distribution to evaluate the integrity of the mitochondrial membranes. In mammalian cells, a major apoptosis activation pathway is the cytochrome c-initiated pathway (Jiang and Wang 2004). Cytochrome c induces a series of biochemical reactions that result in caspase activation and interact with the apoptotic protease activating factor-1 (Apaf-1) in the presence of ATP when released from mitochondria. This interaction induces a conformational change in Apaf-1 that recruits and activates procaspase-9. The complex formed by cytochrome c, Apaf-1, ATP, and procaspase-9 has been termed the "apoptosome." At the same time, procaspase-9 becomes an active caspase-9 (Marsden et al. 2002). Then, caspase-9 activates downstream caspases with a short prodomain, such as caspase-3 and caspase-7 (Pop et al. 2006), which are the key mediators of apoptosis in mammalian cells. In our study, the changes in mitochondrial membrane potential and cytochrome c distribution were suppressed by overexpressing ROP18 in 293T cells. This suppression was dramatically different at 24 and 36 h, but not at 48 h. We hypothesized that ROP18 overexpression in 293T cells suppressed the induced (by ActD) cell apoptosis, maintained cell mitochondrial membrane potential, and prevented mitochondrial cytochrome c leakage.

Hwang et al. (2010) found that $T.$ $gondii$ infection induced host cell overexpression of Bcl-2, a family of proteins serving as major regulators of apoptosis. Bcl-2 promotes cell survival by inhibiting the adapters needed for activation of the proteases (such as caspases) (Yang et al. 1997). Bax is a pro-apoptotic protein of the Bcl-2 family that induces cytochrome c release and caspase activation (Ohtsuka et al. 2004). Our results indicated that overexpressing ROP18 upregulated 293T cell's Bcl-2 coding gene expression and downregulated Bax coding gene expression. The results further confirmed that $T.$ $gondii$ ROP18 manipulates host cell apoptosis via the cytochrome c-initiated pathway.

We speculate that $T.$ $gondii$ ROP18 is a potential factor regulating host cell apoptosis. $T.$ $gondii$ may maintain the integrity of the mammalian cell mitochondrial membrane and hinder the release of cytochrome c, which is the inducer for mammalian cell apoptosis. However, we know that ROP18 is not the only secreted protein that manipulates host cell apoptosis, of which there are a few. Future studies will further research the ROP18 activity in $T.$ $gondii$ to more thoroughly explore the mechanism revealed above.

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