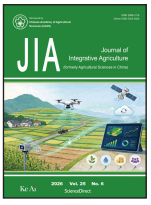
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RESEARCH ARTICLE

PEX5-mediated modulation of apoptotic pathways in response to Newcastle disease virus infection

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Highlights

- Newcastle disease virus (NDV) infection markedly reduces cellular PEX5 expression.
- PEX5 negatively regulates NDV-induced apoptosis.
- The PEX5-Bcl2/BAX axis is essential for NDV-induced apoptosis.

Abstract

Newcastle disease virus (NDV) is a highly lethal and contagious viral pathogen, and it is also a potent oncolytic virus that selectively replicates in tumor cells. NDV demonstrates high replication efficiency in avian and tumor cells, causing various types of cell death, including ferroptosis, necrosis, apoptosis and autophagic cell death, with apoptosis being the most thoroughly studied. Organelles play critical and distinctive roles in the regulation and execution of apoptosis. However, the involvement of peroxisomes, an important organelle that regulates redox balance and lipid biosynthesis, in virus-induced apoptosis remains unclear. Our findings reveal that NDV infection promotes the downregulation of several peroxisome biogenesis factors (*PEXs*) at the mRNA level. Peroxisomal biogenesis factor 5 (PEX5), a critical peroxisomal shuttle protein, was identified to be significantly downregulated at both the mRNA and protein levels. Further, gain- and loss-of-function experiments demonstrated the negative regulation of NDV-induced apoptosis by PEX5. In addition, PEX5 inhibits NDV-induced apoptosis by regulating the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) expression. These findings reveal a novel mechanism by which NDV-induced apoptosis is modulated through the downregulation of PEXs, particularly PEX5, shedding light on the potential role of peroxisome in apoptosis regulation in response to virus infection.

Keywords: NDV, peroxisome, PEX5, Bcl-2, apoptosis

1. Introduction

Newcastle disease virus (NDV), classified as part of the *Orthoavulavirus* genus of the *Paramyxoviridae* family, is a highly contagious and highly lethal avian pathogen that causes significant economic losses to the poultry industry worldwide (Amarasinghe *et al.* 2019; Alexander 2000). NDV exhibits a broad host range, with avian cells serving as its primary natural hosts (Abdullahi *et al.* 2018). The clinical manifestations of Newcastle disease (ND) exhibit considerable variation and are influenced by factors such as the specific virus strains, the avian species affected, and the age of the host, among other determinants. Common clinical symptoms include respiratory distress, gastrointestinal disturbances, neurological signs, and abnormal egg production, etc. (Alexander and Senne 2003). Notably, the velogenic strain of NDV possesses the capability to cause almost 100% mortality in infected chickens within a remarkably brief timeframe (Miller *et al.* 2013). Apart from posing a significant threat to poultry, the oncolytic potential of NDV has been substantiated through studies conducted in cell cultures, experimental animal models, and clinical trials (Alexander and Senne 2003). Studies have reported that NDV effectively kills tumors while exerting minimal side effects on normal cells (Zamarin and Palese 2012;

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Schirrmacher 2015; Cheng *et al.* 2016). NDV can selectively replicate in tumor cells and induce various types of cell death, including ferroptosis, necrosis, apoptosis and autophagic cell death (Lam *et al.* 2011; Sui *et al.* 2017; Li *et al.* 2019; Kalantari *et al.* 2020; Kan *et al.* 2021; Gong *et al.* 2022; Wang *et al.* 2023; Sun *et al.* 2024). Among all these types of cell death, apoptosis is the most extensively studied and is known to play a crucial role in numerous processes such as normal cell turnover, regulation of the immune system, and pathogenic infection (Elmore 2007). It is reported that the entire life cycle process of NDV replication is necessary for cell apoptosis, which activates a series of endogenous and exogenous apoptosis signals to execute cell apoptosis (Elankumaran *et al.* 2006; Ravindra *et al.* 2008a; Bian *et al.* 2011; Morla *et al.* 2019). In addition to tumor cells, numerous studies have confirmed that NDV induced apoptosis in chicken cells and tissues, thereby indicating the critical role of apoptosis in the pathogenicity of NDV (Ravindra *et al.* 2008b).

Apoptosis is controlled by the regulation of the balance between anti-apoptotic and pro-apoptotic proteins of the B-cell lymphoma-2 (Bcl-2) family, the initiator caspases (caspases-8, -9 and -10) and effector caspases (caspases-3, -6, and -7) (Bertheloot *et al.* 2021). The final reaction of apoptosis culminates in several hallmark events that collectively ensure the controlled and efficient dismantling of the cell (Tang *et al.* 2015). The occurrence of apoptosis is mainly divided into extrinsic and intrinsic pathways (Riedl and Salvesen 2007). The extrinsic pathway is activated through the engagement of membrane receptors (Bertheloot *et al.* 2021). The intrinsic pathway is primarily characterized by the activation of the pro-apoptotic proteins Bcl-2-Associated X (BAX) and Bcl-2 antagonist/killer (BAK), which are members of the Bcl-2 family (Chipuk *et al.* 2010). The activation of BAX and BAK is regulated by anti-apoptotic proteins, such as Bcl-2, and pro-apoptotic proteins of the Bcl-2 family (Chipuk *et al.* 2010). Moreover, the activation of BAX and BAK form pores on the outer membrane of the mitochondria and allow cytochrome-c to be released into the cytoplasm (Kuwana *et al.* 2002; Bleicken *et al.* 2014). Cytochrome-c binds to the Apaf-1 protein, thereby recruiting the caspase-9 precursor to form an apoptosome which activates caspase-9 through autoproteolytic cleavage and initiates the caspase cascade (Li *et al.* 1997; Zou *et al.* 1997; Srinivasula *et al.* 1998; Cain *et al.* 1999).

Organelles play critical and distinctive roles in the regulation and execution of apoptosis, particularly intrinsic apoptosis, thus ensuring that cells can safely and efficiently eliminate themselves without causing damage to surrounding tissues (Lemasters 2005; Cheng and Lane 2010; Wu *et al.* 2023). For example, mitochondria are central to apoptosis, particularly in the intrinsic pathway (Nguyen *et al.* 2023). Upon receiving apoptotic signals, mitochondria release cytochrome-c into the cytosol. This release is regulated by Bcl-2 family proteins and leads to the formation of the apoptosome complex, subsequently activating caspase-9 and downstream effector caspases (caspases-3, -6, and -7) and culminating in cell death (Elmore 2007). The endoplasmic reticulum (ER), lysosomes, and the Golgi apparatus have also been reported to be directly or indirectly involved in the process of apoptosis (Machamer 2015; Solano-Galvez *et al.* 2018; Li *et al.* 2019).

Peroxisomes are closed single-membrane organelles that play crucial roles in cellular and metabolic homeostasis, including redox balance and the biosynthesis of vital lipids (Okumoto *et al.* 2020). Specifically, peroxisomes contain antioxidant enzymes like catalase and peroxidase that neutralize hydrogen peroxide, a harmful byproduct of metabolic processes, thereby protecting the cell from oxidative damage (Lismont *et al.* 2019). Additionally, they play a key role in the β-oxidation of very-long-chain fatty acids (VLCFAs) (Alexander and Senne 2003). Peroxisomes are involved in extensive communication with other organelles, including mitochondria, ER, and lysosomes, which can impact the activation of apoptosis (Diaz *et al.* 2021). Dysfunction in peroxisomes has been linked to a range of pathological conditions, including increased ER stress and mitochondrial dysfunction, which can subsequently elevate cell death rates through apoptosis. For example, peroxisome deficiency induces mitochondrial fragmentation and enhances stress-induced caspase activation and apoptosis (Tanaka *et al.* 2019).

The function of peroxisomes is closely influenced by their size, number, and morphology, all of which are affected by environmental stimuli and exhibit great plasticity (Farre *et al.* 2019). Peroxisome biogenesis is a rather complex and strictly regulated biological process in which a series of peroxisome biogenesis factors (peroxins, PEXs) are specifically involved (Fransen *et al.* 2012). Among these, PEX5 is an important shuttle protein of peroxisomes, which transports peroxisomal matrix proteins from the cytoplasm to the peroxisomes (Skowyra and Rapoport 2022). After binding to matrix proteins, PEX5 binds to the adapter molecules on the peroxisome membrane and then releases the cargo to enter the next cycle (Platta *et al.* 2009; Jiang *et al.* 2024). Furthermore, PEX5 is an important cargo molecule for peroxisome-selective autophagy (pexophagy) (Zhang *et al.* 2015). When cells are stimulated by oxidative stress or starvation, a large amount of intracellular reactive oxygen species (ROS) accumulates; in turn, this activates the ataxia telangiectasia-mutated gene (ATM). The activated ATM causes PEX5 to be ubiquitinated and recognized by autophagy receptors p62, thereby triggering pexophagy (Zhang *et al.* 2015; Sargent *et al.* 2016). Evidence suggests that apoptosis can be regulated by PEX5. For example, decreasing the expression of PEX5 has been reported to inhibit pexophagy but promote apoptosis (Tanaka *et al.* 2019; Wen *et al.* 2020; Jiang and Okazaki 2022; Liu *et al.* 2023).

Further, increasing evidence indicates that apoptosis plays a rather important role in the pathogenic process and oncolytic activity of viruses. In this study, we found the extensive regulation of the expression of *PEX* genes during NDV infection. Gain- and loss-of-function experiments demonstrated the negative regulation of apoptosis by PEX5. In addition, PEX5 inhibits NDV-induced apoptosis by regulating Bcl-2 expression. These findings shed light on the mechanism of peroxisome-mediated apoptosis regulation in response to virus infection.

2. Materials and methods

2.1. Cells, viruses, and reagents

Adenocarcinomic human alveolar basal epithelial (A549) cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). NDV strains Herts/33 were obtained from the China Institute of Veterinary Drug Control (Beijing, China). LinBio Cell Counting Kit-8 (CCK-8) were purchased from LINDE SCIENTIFIC (LC3001, China). Primary antibodies, including mouse anti-nucleocapsid protein (NP) monoclonal antibody, were prepared in our laboratory (Sun *et al.* 2014). Anti-poly ADP-ribose polymerase (PARP) (9542S), anti-caspase-8 (4927), anti-cleaved caspase-8 (Asp374) (8592), anti-caspase-9 (9502), anti-cleaved caspase-9 (Asp353) (9509), anti-caspase-3 (9662S), anti-cleaved caspase-3 (Asp175) (9664T), anti-PEX5 (83020), anti-Bax (2772T), anti-HA (C29F4) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-2 (ab32124) was purchased from Abcam (Cambridge, MA, USA). Anti-β-actin (AC026) was purchased from ABclonal (Wuhan, China). Secondary antibodies, including horseradish peroxidase-conjugated (HRP) goat anti-mouse or goat anti-rabbit antibodies, goat anti-mouse Alexa Fluor® 488 (A-11029), and goat anti-rabbit Alexa Fluor® 594 (A-11037) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). In addition, the Annexin V-FITC Apoptosis Detection Kit (C1062L) was purchased from Beyotime (Shanghai, China).

2.2. RNA-seq

The total RNA of mock and NDV-infected cells was extracted using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. The quantity and purity of the total RNA were analyzed using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA, 5067-1511). After the sample RNA passed the quality inspection, it was sent to Lianchuan Biotech (China) for construction of high-quality RNA samples with RIN number>7.0 sequencing library; 2×150 bp paired-end sequencing (PE150) was performed on Illumina Novaseq™ 6000 (LC-Bio Technology CO., Ltd., Hangzhou, China). Transcriptome sequencing (RNA-seq) data were compared to the human genome in National Center for Biotechnology Information (NCBI), and gene expression levels were estimated by read counts. To make them comparable between different genes or samples, read numbers were converted into counts to normalize gene expression.

2.3. RNA isolation and real-time quantitative PCR

Total intracellular RNA was extracted from NDV-infected and NDV-uninfected cells (multiplicity of infection (MOI)=1) using TRIzol reagent, and RNA samples (2 μg each) were then reverse-transcribed into cDNA using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China, AE311-03) and HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, NanJing, China, R223-01). The resulting cDNA was subsequently used as a template for a Power Green qPCR Mix assay (Dongsheng Biotech, Guangzhou, China, P2092a). Further, each sample's relative mRNA expression levels were normalized to the expression of β-actin mRNA. All samples were assayed 3 times and these assays were performed in an ABI 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA). The designed qRT-PCR primers were listed in Appendix A.

2.4. Western blotting analysis

Western blotting analysis was performed in accordance with our previously described protocol (Kan *et al.* 2021). Briefly, cell samples were lysed with a RIPA buffer and boiled at 100°C for 10 min. Subsequently, samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Whatman, NC-a101-b105). After blocking with 3% skim milk for 1.5 h at room temperature, the membranes were incubated with the appropriate primary antibodies overnight at 4°C. After washing with 1×TBST 5 times, the membrane was incubated with the corresponding secondary antibody at room temperature for 2 h. Then, after washing the membranes 5 times in TBST, the immunoblot signals were developed with Western ECL substrate (Bio-Rad, CA, USA) and detected by the Bio-Rad ChemiDoc imaging system (Bio-Rad). The band intensity of the blots was analyzed using ImageJ software.

2.5. Apoptosis detection

After A549 wild type (WT) cells, *PEX5*$^{-/-}$ and *PEX5* stable expression cell lines were mock-treated or infected with NDV at an MOI of 1 for 24 h. Cell samples were collected and resuspended in 195 μL binding buffer and then stained with 5 μL of annexin V-FITC and 10 μL of propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit in accordance with the manufacturer's instructions. Following incubation on ice for 40 min, the samples were analyzed *via* flow cytometry.

2.6. Indirect immunofluorescence assay (IFA)

Cells grown on coverslips were mock-treated or infected with NDV at an MOI of 1 for 24 h. The cells were washed 3 times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature for 20 min, washed three times with PBS, and then permeabilized with 0.5% Triton X-100 at room temperature for 10 min. Then, after washing 3 times with PBS, the cells were blocked with 3% bovine serum albumin (BSA) at 37°C for 30 min and incubated with primary antibodies at 37°C for 2 h. Thereafter, the cells were washed 3 times with PBS and incubated with anti-mouse or anti-rabbit antibodies (Thermo Fisher Scientific) conjugated to Alexa Fluor 488 or 594 at 37°C for 2 h. Subsequently, the slides were mounted using mounting medium containing DAPI and then air-dried at room temperature in a dark place. Finally, fluorescence images were examined using a confocal fluorescence microscope (Zeiss LSM880, Germany).

2.7. Construction of PEX5 stable expression and knock-out cell lines

The design of cloning primers was based on the reference

sequence of the National Center for Biotechnology Information (NCBI), NM_001351132.2, for human *PEX5*. The *PEX5* gene containing an HA tag at the C-terminus is cloned into the phage-bsd plasmid. The forward primer was ATGGACTACAAAGACCATGACGGTG CAATGCGGGAGCTGGTGGA; the reverse primer was TTAAGCGTAATCTGGAACATCGTATGGGTACTGGGG CAGGCCAAACATAG. In addition, *PEX5* sgRNA is designed using the Synthego CRISPR Design Tool (https://design.synthego.com) and then cloned into the Lenti-CRISPR-V2 vector. The sequences of sgRNAs are sgRNA *PEX5* 1#: ATTTCCCTTCTGGCAGTTGG; sgRNA *PEX5* 2#: GTTGGTGGCTGAATTCCTGC; and sgRNA *PEX5* 3#: GGGGTGCATTCTGGTCCTGC. Briefly, the recombinant stably expressed *PEX5* and sgRNA recombinant plasmids were packaged with PAX and PMD2G respectively, and then the A549 cells were infected with the lentivirus. After 3 rounds of selection with 10 μg mL^{-1} puromycin (Thermo Fisher Scientific), the surviving cells were subjected to limiting dilution for subcloning. Finally, Western blotting was used to verify the protein expression level of *PEX5*.

2.8. RNA interference

The siRNAs targeting *Bcl-2* (si*Bcl-2*) and the negative control (siRNA-NC) were designed by GenePharma (Shanghai, China). The siRNA with 100 pmol was transfected into WT and *PEX5*$^{-/-}$ A549 cells using Lipofectamine 2000 (Invitrogen) for 48 h according to the manufacturer's instructions; this was followed by Western blotting to verify the efficacy of the interference. The transfected WT and *PEX5*$^{-/-}$ A549 cells were used for subsequent experiments. The si*Bcl-2* sequences are (a) GAGAACAGGGUACGAUAACTT; (b) GGGAGGAUUGUGGCCUUCUTT; and (c) GUGGAUGAC UGAGUACCUGTT.

2.9. Evaluation of cell viability

Cell viability was evaluated using the CCK-8 assay in accordance with the manufacturer's instructions. Briefly, cells with or without knocking out *PEX5* were seeded into a 96-well plate and incubated for 48 h. These cells were then treated with 10 μL of CCK-8 reagents for an additional 1 h at 37°C in the dark. The absorbance was measured in a microplate reader at a wavelength of 450 nm.

2.10. Statistical analysis

Statistical differences were determined using the GraphPad Prism 8.0 software (USA). All experiments were performed in triplicate, and the data were analyzed using a Student's *t*-test (*t*-test). A *P*-value of less than 0.05 was considered statistically significant in all experiments.

3. Results

3.1. NDV decreases the expression of PEX5 in A549 cells

To investigate the overall host gene expression profile during NDV infection, RNA-seq was performed in NDV-infected A549 cells. The transcriptome revealed that NDV infection reduces the expression of a large number of peroxisome biosynthesis-related genes, such as *PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX11A*, and *PEX11G* (Fig. 1-A). Similar expression profiles of *PEX* genes could also be observed upon infection with other viruses, such as porcine epidemic diarrhea virus (PEDV), Zika virus (ZIKV), and hepatitis C virus (HCV) (Lupberger *et al.* 2019). To further confirm the impact of NDV on *PEX* gene expression, quantitative PCR was conducted to validate the expression of the aforementioned genes. As expected, NDV infection significantly downregulated the expressions of *PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX11A*, and *PEX11G* (Fig. 1-B). Among these the mRNA abundance of *PEX5* exhibited the most pronounced decrease following NDV infection. As PEX5 is the only known import receptor that crosses the membrane and functions as a major importing factor responsible for transporting peroxisomal matrix proteins into peroxisomes (Skowyra and Rapoport 2022), we mainly focused on PEX5 to investigate its role in NDV infection. Therefore, the protein level of PEX5 was validated in NDV-infected cells. The results revealed that NDV significantly reduced PEX5 protein levels in a time- and dose-dependent manner (Fig. 1-C–F). Collectively, these results indicated that NDV infection reduces the expression of *PEX* genes, particularly the matrix shuttle protein gene *PEX5*, which probably induces the dysfunction of peroxisomes.

3.2. Overexpression of PEX5 inhibits NDV-induced apoptosis

A previous report has proven the potential role of PEX5 in apoptosis and pexophagy (Zhang *et al.* 2015; Liu *et al.* 2023). However, the function of PEX5 in the pathogenesis of virus infection has not been elucidated. Since apoptosis plays an important role in NDV-induced cell death (Elankumaran *et al.* 2006; Ravindra *et al.* 2008a; Li *et al.* 2019), we questioned whether PEX5 is involved in NDV-induced apoptosis. To verify this hypothesis, the A549 cell line that stably expresses *PEX5* was generated (Fig. 2-A). The stable expression of *PEX5* had no effect on cell viability (Fig. 2-B). Then, annexin V-FITC and propidium iodide (PI) apoptosis-detection kits were used to detect apoptotic cells. The results revealed that *PEX5* overexpression significantly decreased NDV-induced apoptosis compared with WT cells (Fig. 2-C and D). In comparison, the background apoptosis level was not affected (Fig. 2-C and D). Accordingly, the cleavage of caspases-3, -8, and -9 and PARP induced by NDV was found to be decreased in *PEX5* overexpressed cells compared with wild type (WT) cells (Fig. 2-E). Additionally, immunostaining also revealed that cells stably expressing *PEX5* exhibited a lower apoptosis level than WT cells upon NDV infection, as indicated by the weaker fluorescence signal of cleaved caspases-3 and-8 (Fig. 2-F and G). These results suggest that overexpression of *PEX5* significantly inhibits apoptosis induced by NDV.

3.3. Knockout of PEX5 promotes NDV-induced apoptosis

To further explore the effect of PEX5 in NDV-induced apoptosis, we constructed a *PEX5* gene knockout A549 cell line (*PEX5*$^{-/-}$). The results confirmed the knockout of *PEX5* at the protein level without affecting the cell viability (Fig. 3-A

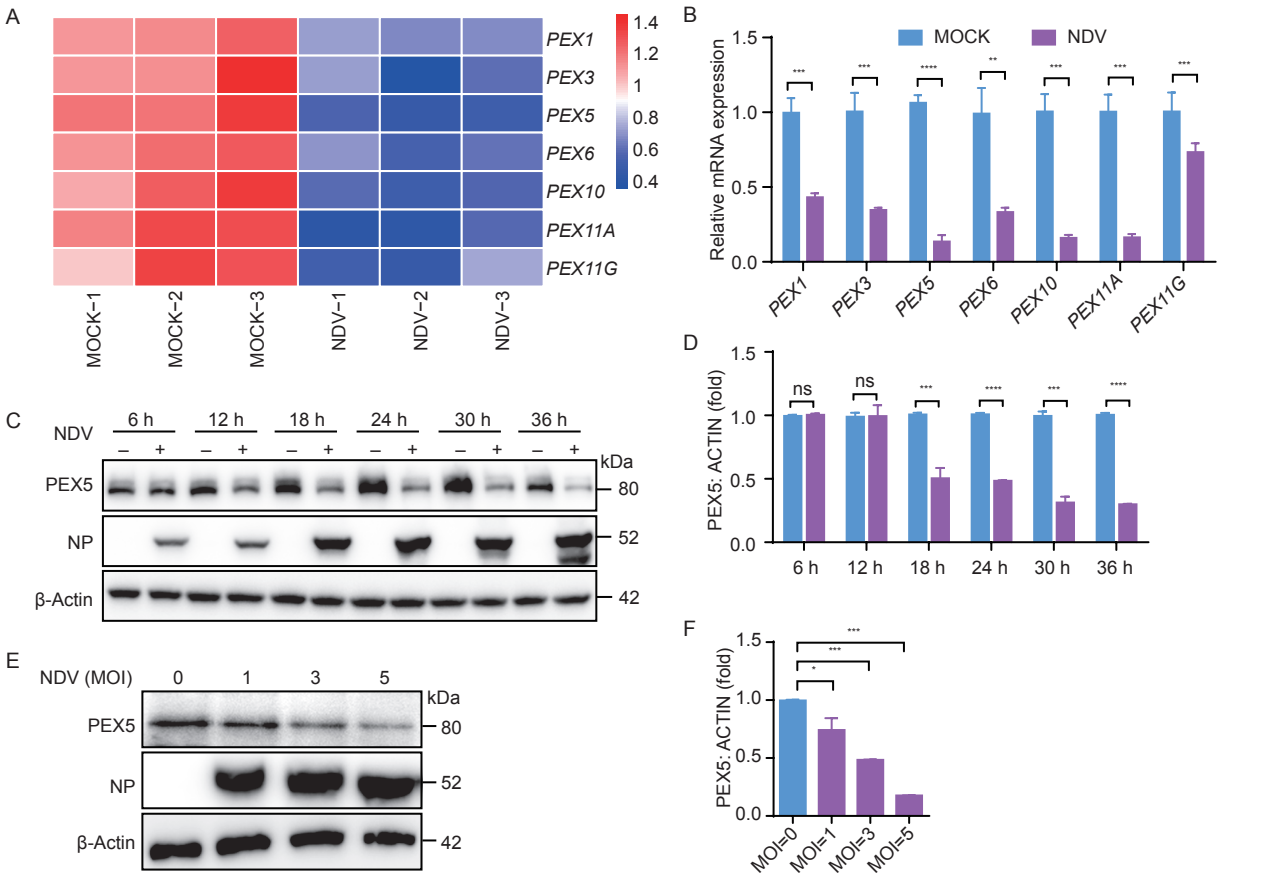


Fig. 1 Newcastle disease virus (NDV) decreases the expression of PEX5 in A549 cells. A, Heat map of differential expression of genes related to peroxisome biogenesis (*PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX11A*, and *PEX11G*) in mock-treated and NDV-infected A549 cells. B, validation of peroxisome biogenesis-related genes (*PEX1*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX11A*, and *PEX11G*) expression by RT-qPCR. C, A549 cells were mock treated or infected with NDV for 6, 12, 18, 24, 30, and 36 h (multiplicity of infection (MOI)=1). PEX5, NDV, nucleocapsid protein (NP), and β-actin protein levels were determined by Western blotting. D, representative results are depicted, with graphs representing the band intensity ratios of PEX5/β-actin. E, A549 cells were mock treated or infected with NDV for 24 h (MOI=1, 3, and 5). PEX5, NP, and β-actin protein levels were determined by Western blotting. F, representative results are presented, with graphs representing the band intensity ratios of PEX5/β-actin. Data are mean±SE; all data are from three independent experiments; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; ns, $P>0.05$.

and B). As expected, flow cytometric analysis of annexin V/PI staining revealed that the knockout of *PEX5* did not increase the basal level of apoptosis (Fig. 3-C and D). However, NDV-induced apoptosis was significantly increased in *PEX5*$^{-/-}$ cells, compared with WT cells (Fig. 3-C and D). The detection of the main biochemical indicators of apoptosis revealed that the knockout of *PEX5* exacerbated the occurrence of apoptosis upon NDV infection, which was evidenced by the pronounced increase of the cleaved production of caspases-3, -8, and -9 and PARP (Fig. 3-E). Not surprisingly, immunostaining also revealed that more cells underwent apoptosis in *PEX5*$^{-/-}$ cells upon NDV infection, as indicated by the levels of cleaved caspases-3 and -8 (Fig. 3-F and G). Collectively, the above gain- and loss-of-function experiments clearly demonstrated the negative regulation of NDV-induced apoptosis by PEX5.

3.4. PEX5 regulated Bcl-2 expression upon NDV infection

BAX is a pro-apoptotic factor that participates in the mitochondrial apoptosis pathway, while Bcl-2 is a major anti-apoptotic factor and can inhibit BAX-induced apoptosis (Rahmani *et al.* 2018). The BAX/Bcl-2 ratio represents a rheostat that determines cell susceptibility in response to an apoptotic stimulus (Gao and Wang 2009). To explore the mechanism by which PEX5 regulated NDV-induced apoptosis, the expression of BAX and Bcl-2 was detected in *PEX5* KO and overexpressed cells. In WT cells, the BAX/Bcl-2 ratio was increased under NDV infection, thereby indicating that NDV probably induced apoptosis by the inhibition of Bcl-2 (Fig. 4-A–D, lane/column 1 *vs*. 2). Interestingly, the knockout of the *PEX5* gene significantly exacerbated the BAX/Bcl-2 ratio (Fig. 4-A and B, lane/column 2 *vs*. 4), while the stable expression of *PEX5* inhibited the degradation of Bcl-2 induced by NDV (Fig. 4-C and D, lane/column 2 *vs*. 4). In contrast, in the absence of a viral infection, there was no significant difference in the ratio of BAX/Bcl-2 between WT and either *PEX5* KO or overexpressed cells, respectively (Fig. 4-A–D, lane/column 1 *vs*. 3). These results indicate that PEX5 likely regulates NDV-induced apoptosis through the BAX/Bcl-2 ratio.

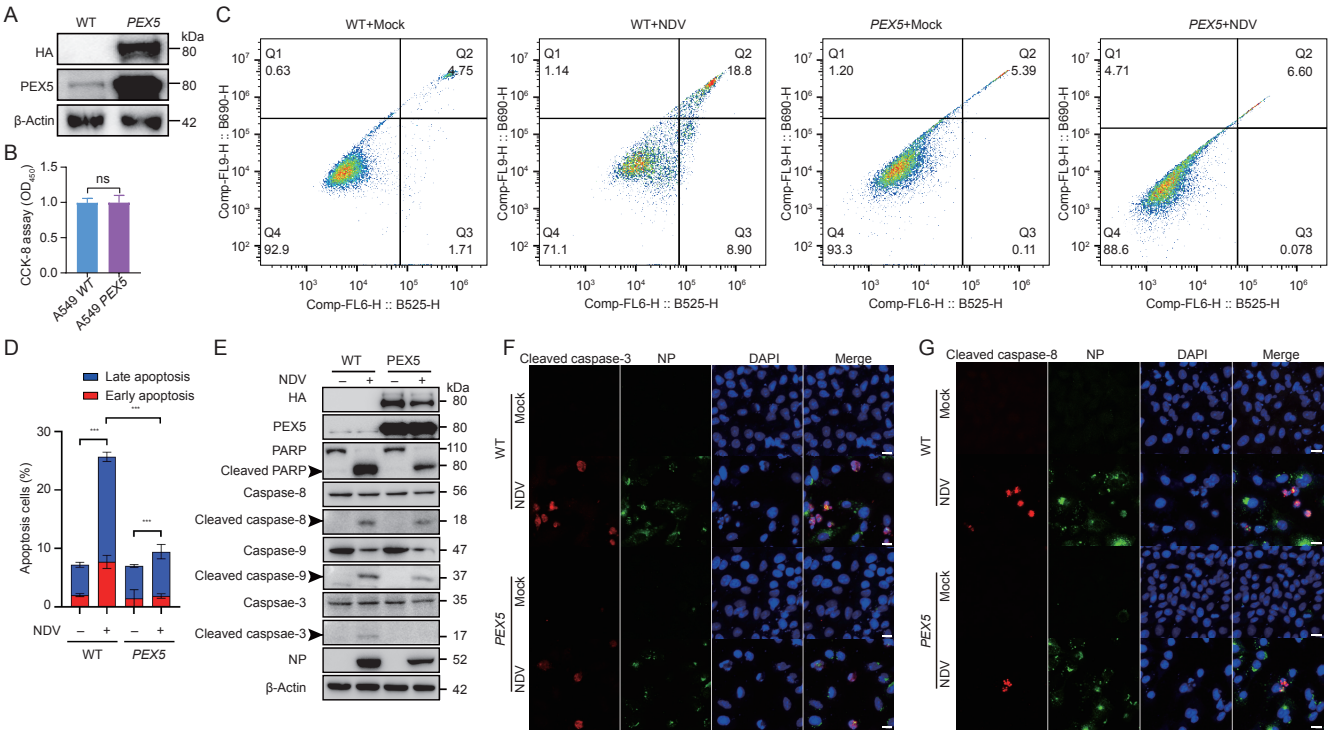


Fig. 2 PEX5 overexpression inhibits Newcastle disease virus (NDV)-induced apoptosis. A, Western blotting analysis of PEX5 and HA protein levels in A549 cells and stable expression vector control (HA), or HA-*PEX5*. B, CCK-8 detection kit was used to detect the cell viability of A549 wild type (WT) cells and stable expression *PEX5* cell lines. C, detection of NDV-induced apoptosis by using Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) in accordance with the manufacturer's instructions. A549 WT cells and stable expression HA-*PEX5* cells were infected with mock-infected or NDV (multiplicity of infection (MOI)=1), stained with annexin V/PI, and analyzed with flow cytometry at 24 hours post infection (hpi). D, statistical analysis of NDV-induced apoptosis in A549 WT cells and stable expression *PEX5* cells. E, Western blotting analysis of poly ADP-ribose polymerase (PARP); cleaved PARP; caspases-3, -8, and -9; cleaved caspase-3, -8, and -9; nucleocapsid protein (NP); and β-actin in WT cells; and cells with a stable expression of *PEX5* after NDV infection for 24 h (MOI=1). F and G, WT cells and cells with a stable expression of *PEX5* were mock-treated or infected with NDV for 24 h (MOI=1) and then immunostained with antibodies that detected cleaved caspase-3 (F) and -8 (G), respectively. Scale bar=10 μm. Data are mean±SE; all data are from three independent experiments; ***, $P<0.001$; ns, $P>0.05$.

3.5. Bcl-2 depletion alleviated NDV-induced apoptosis in *PEX5*$^{-/-}$ cells

In order to further verify the role of Bcl-2 in PEX5-mediated apoptosis upon NDV infection, *Bcl-2* was knocked down in WT and *PEX5*$^{-/-}$ cells, respectively, followed by the detection of NDV-induced apoptosis. The interference efficiency of si*Bcl-2* was first verified by Western blotting and si*Bcl-2* 3# was selected for further study (Fig. 5-A). In a scramble siRNA group, the knockout of *PEX5* significantly promoted NDV-induced cleavage of caspases-3, -8, and -9 and PARP (Fig. 5-B, lane/column 2 *vs*. 6), which is consistent with the results described above. Importantly, compared with the scramble siRNA group, in the si*Bcl-2* group, the knockout of *PEX5* only slightly increased the NDV-induced cleavage of PARP and caspase-8, and even decreased the NDV-induced cleavage of caspases-3 and -9 (Fig. 5-B, lane 4 *vs*. 8), thereby indicating that *Bcl-2* knockdown alleviated NDV-induced apoptosis in *PEX5*$^{-/-}$ cells compared with WT cells. The flow cytometric analysis of Annexin V/PI staining further demonstrated that *PEX5* knockout significantly promoted apoptosis in scramble siRNA group (Fig. 5-C and D, column 2 *vs*. 6) but not in the si*Bcl-2* group (Fig. 5-C and D, column 4 *vs*. 8). These results clearly demonstrated the critical role of Bcl-2 in PEX5-mediated apoptosis upon NDV infection. Taken together, our study reveals the potential role of PEX5 in virus-induced apoptosis. Specifically, NDV infection induced the degradation of PEX5, which promotes apoptosis by regulating the BAX/Bcl-2 ratio (Fig. 4-E).

4. Discussion

NDV demonstrates high replication efficiency in tumor and avian cells, particularly those derived from chickens. This efficient viral propagation is facilitated by the aberrant antiviral responses of tumor cells and the inherent susceptibility of chicken cells, which provides a unique platform for studying pathogenicity and the potential therapeutic applications of NDV (Gao and Wang 2009). The cumulative effects of interferon signaling in tumor cells could also lead to a higher apoptotic effect, which may be the reason for the tumor-selectivity of NDV (Gao and Wang 2009). NDV replicates rapidly, thereby causing cell damage and death by inducing several types of cell death, such as apoptosis, necrosis, ferroptosis, etc. These different types of cell death caused by NDV may be influenced by the state of the host cell and the particular virus strain. Nevertheless, extensive studies have proven that apoptosis is one of the primary mechanisms

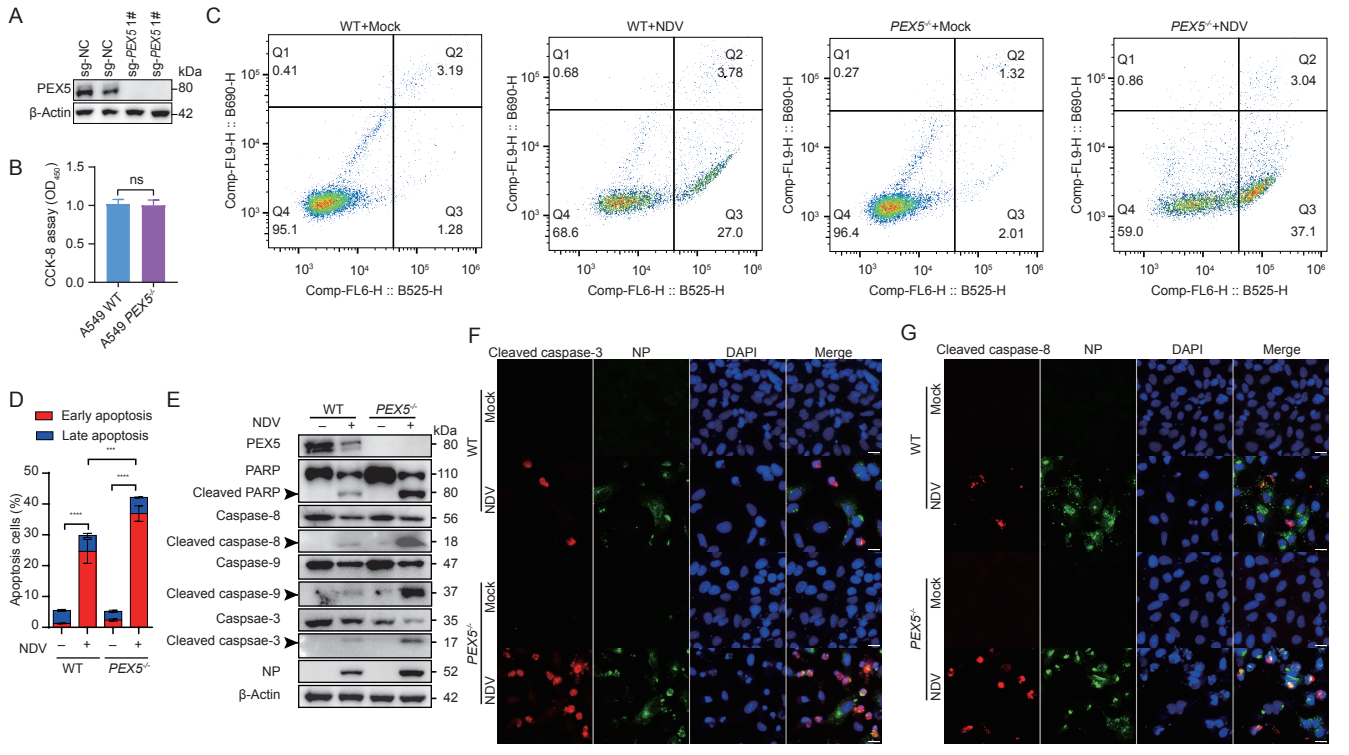


Fig. 3 Knockout of *PEX5* promotes Newcastle disease virus (NDV)-induced apoptosis. A, Western blotting analysis of PEX5 protein expression in wild type (WT) and *PEX5*$^{-/-}$ A549 cells. B, CCK-8 detection kit was used to detect the cell viability of A549 WT cells and PEX5 knockout cell lines. C, detection of NDV-induced apoptosis by using the Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) in accordance with the manufacturer's instructions. WT and *PEX5*$^{-/-}$ A549 cells were mock-treated or infected with NDV for 24 h (multiplicity of infection (MOI)=1), stained with Annexin V/PI, and analyzed using flow cytometry. D, statistical analysis of NDV-induced apoptosis in WT and *PEX5*$^{-/-}$ A549 cells. E, Western blotting analysis of poly ADP-ribose polymerase (PARP); cleaved PARP; caspases-3, -8, and -9; cleaved caspases-3, -8, and -9; nucleocapsid protein (NP); and β-actin in WT and *PEX5*$^{-/-}$ A549 cells after NDV infection for 24 h (MOI=1). F and G, WT and *PEX5*$^{-/-}$ A549 cells were mock-treated or infected with NDV for 24 h (MOI=1) and then immunostained with antibodies that detected cleaved caspases-3 (F) and -8 (G), respectively. Scale bar=10 μm. Data are mean±SE; all data are from three independent experiments; ***, P<0.001, ****, P<0.0001; ns, P>0.05.

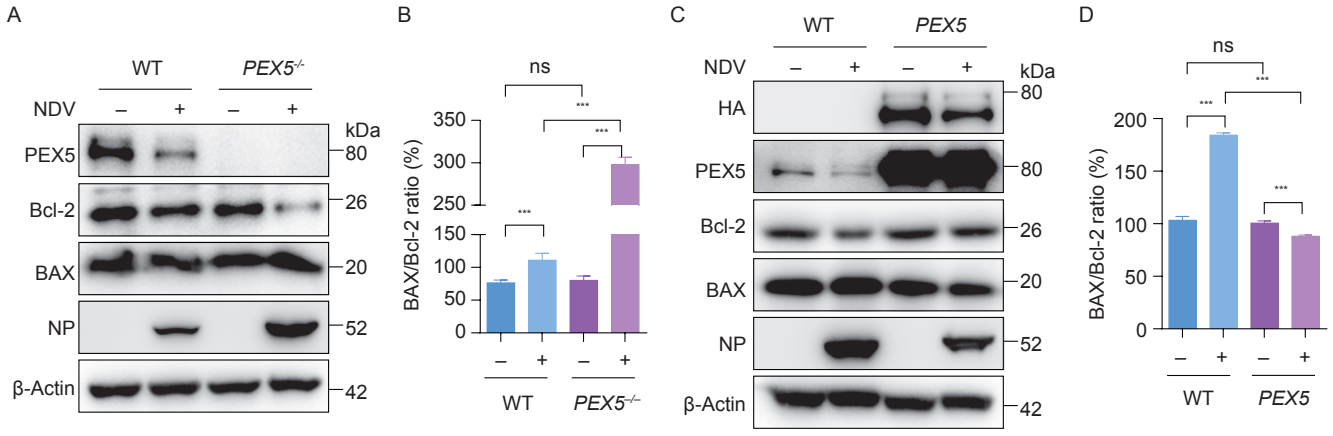


Fig. 4 PEX5 regulated *Bcl-2* and *BAX* expression upon Newcastle disease virus (NDV) infection. A, Western blotting analysis of PEX5, Bcl-2, Bcl-2-associated X (BAX), nucleocapsid protein (NP), and β-actin in wild type (WT) and *PEX5*$^{-/-}$ A549 cells after NDV infection for 24 h (multiplicity of infection (MOI)=1). B, statistical analysis of the ratio of BAX to Bcl-2 in Fig. 4-A. C, Western blotting analysis of PEX5, Bcl-2, BAX, NP, and β-actin in WT cells and cells with a stable expression of *PEX5* after NDV infection for 24 h (MOI=1). D, statistical analysis of the ratio of BAX to Bcl-2 in Fig. 4-C. Data are mean±SE; all data are from three independent experiments; ***, P<0.001; ns, P>0.05.

of cell death induced by NDV (Li *et al.* 2019). In this study, we demonstrated that NDV infection induced degradation of PEX5, the peroxisomal shuttle protein, which promotes apoptosis by regulating Bcl-2 expression.

Apoptosis is a process in which cells die in response to signals they generate in order to eliminate damaged or

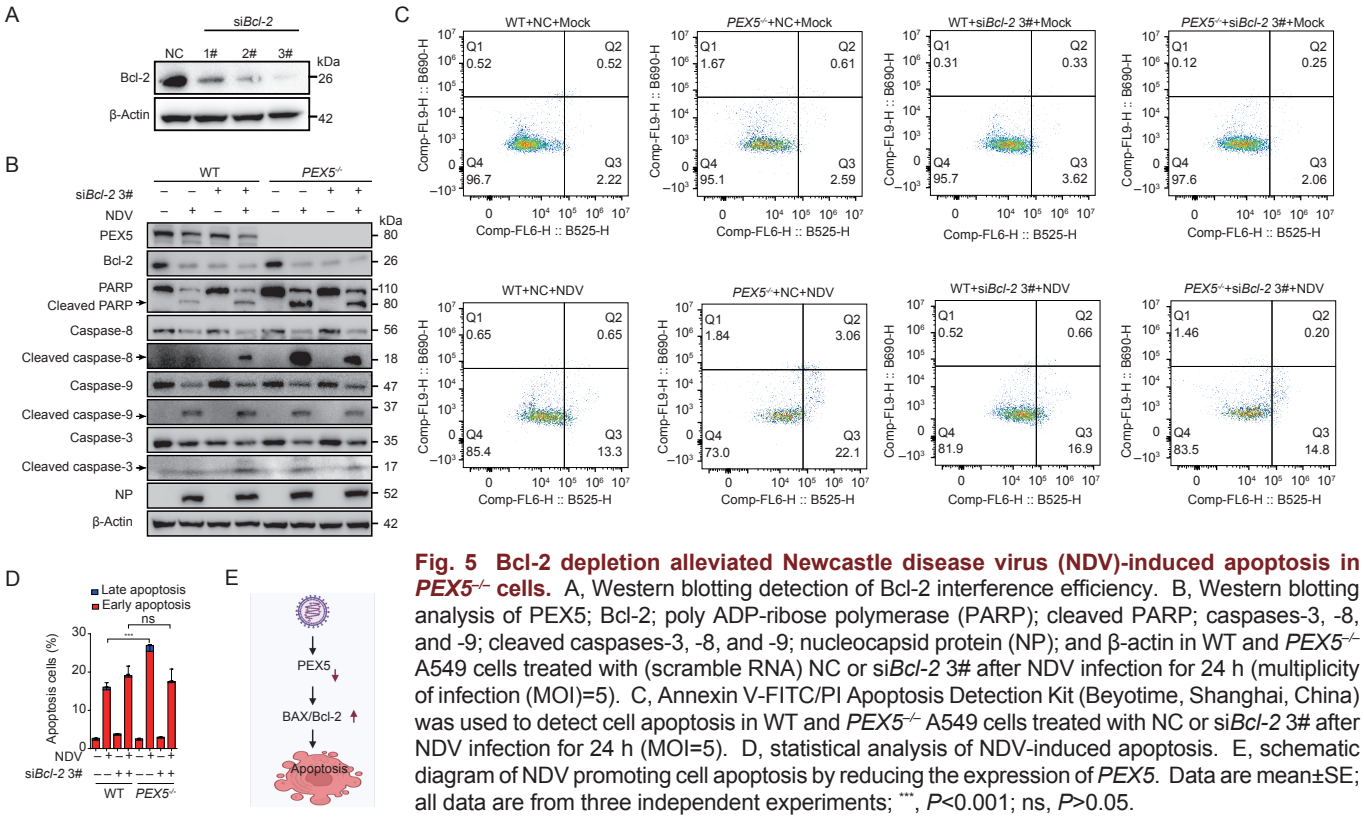


Fig. 5 Bcl-2 depletion alleviated Newcastle disease virus (NDV)-induced apoptosis in *PEX5*$^{-/-}$ cells. A, Western blotting detection of Bcl-2 interference efficiency. B, Western blotting analysis of PEX5; Bcl-2; poly ADP-ribose polymerase (PARP); cleaved PARP; caspases-3, -8, and -9; cleaved caspases-3, -8, and -9; nucleocapsid protein (NP); and β-actin in WT and *PEX5*$^{-/-}$ A549 cells treated with (scramble RNA) NC or si*Bcl-2* 3# after NDV infection for 24 h (multiplicity of infection (MOI)=5). C, Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) was used to detect cell apoptosis in WT and *PEX5*$^{-/-}$ A549 cells treated with NC or si*Bcl-2* 3# after NDV infection for 24 h (MOI=5). D, statistical analysis of NDV-induced apoptosis. E, schematic diagram of NDV promoting cell apoptosis by reducing the expression of *PEX5*. Data are mean±SE; all data are from three independent experiments; ***, $P<0.001$; ns, $P>0.05$.

dysfunctional cells (Nam *et al.* 2019; Vogel *et al.* 2019). This process plays an important role in treating cancer and tumors. It has been reported that NDV infection can induce apoptosis through various mechanisms. For example, NDV infection promotes apoptosis by stimulating the production of interferon (Ginting *et al.* 2019). In addition, NDV infection mediates cell apoptosis through the C/EBP homology protein (CHOP), inositol-requiring enzyme 1 α (IRE1α)-X-box binding protein 1 (XBP1), IRE1-thioredoxin-interacting protein (TXNIP) and c-Jun N-terminal kinase (JNK) signaling pathways (Li *et al.* 2019). NDV can also promote apoptosis by upregulating the expression of p53 and BAX and reducing the expression of *Bcl-2* (Ravindra *et al.* 2009). Here, we identified PEX5 as the upstream regulatory molecule of BAX/Bcl-2-mediated apoptosis during an NDV infection.

Further, the knockdown of *PEX5* has been reported to increase endogenous apoptosis in HepG2 cells (Wang *et al.* 2017; Liu *et al.* 2023). However, the background level of apoptosis in A549 cells was not affected by either *PEX5* KO or stable overexpression. This difference may be due to the use of different cell lines. Moreover, the sensitivity of tumor cells to apoptosis can vary significantly based on factors such as cell origin, genetic mutations, and signaling pathways, including the expression patterns of Bcl-2 family proteins (Gao and Wang 2009). Nevertheless, our results clearly demonstrated the negative regulation of apoptosis by PEX5 in the course of NDV infection. To the best of our knowledge, this is the first case report on the regulation of virus-induced apoptosis by PEX5. We speculate that this mechanism may also be shared by other viruses, or at least other oncolytic viruses, which remains to be studied.

A significant down-regulation of *PEX5* expression was observed during NDV infection as cells must remove excess or damaged peroxisomes to maintain their homeostasis (Walker *et al.* 2018). As an important shuttle protein of peroxisomes, the expression of *PEX5* is tightly regulated during the biogenesis of peroxisomes. Ubiquitin modification can target PEX5 for recycling or degradation (Thoms and Erdmann 2006; Wang *et al.* 2017) — for example, in yeast, ubiquitin-conjugating enzyme 4 (UBC4) and PEX2 polyubiquitinate PEX5 targets PEX5 for proteasomal degradation (Thoms and Erdmann 2006). In addition, under oxidative stress, the PEX5 ubiquitination also triggers pexophagy mediated by p62. During this process, PEX5 was degraded through the autophagic pathway (Zhang *et al.* 2015). In yeast, PEX5 and PEX7 can be degraded by a new selective autophagy pathway, which is independent of pexophagy (Wang *et al.* 2020). Overall, these reports have confirmed that the regulation of *PEX5* expression primarily occur at the protein level, which may also account for the NDV-induced degradation of PEX5. Notably, the mRNA abundance of *PEX5*, as well as several other *PEX* genes, decreased following NDV infection; this suggests the possible regulation of *PEX5* at the mRNA level, which needs to be further researched.

Organelles, particularly mitochondria, function as the critical platform or hub for the execution of apoptosis, especially intrinsic apoptosis (Wu *et al.* 2023). Mitochondria are both the source and target of ROS, which have been confirmed to be involved in apoptosis regulation (Simon *et al.* 2000; Sinha *et al.* 2013; Yee *et al.* 2014). In addition, peroxisomes are important sites for ROS production

and clearance and play an important role in maintaining cellular redox homeostasis. When exposed to excessive ROS, PEX5 was found to be phosphorylated at S141 and monoubiquitylated at K209, which is targeted by p62 for peroxisome selective autophagy, also called pexophagy (Zhang *et al*. 2015). Our results revealed the obvious degradation of PEX5 at the protein level during NDV infection. Moreover, it has been reported that NDV induces oxidative stress in tissue and cells (Rehman *et al.* 2018; Shokeen and Kumar 2024). Therefore, there is a high possibility that NDV infection causes oxidative stress and subsequently induces pexophagy through PEX5. The crosstalk between apoptosis and autophagy has been widely reported (Maiuri *et al.* 2007; Gordy and He 2012). Since mitochondria are central to the death of various cells, including apoptosis, mitophagy is certainly an important regulatory mechanism for apoptosis (Wanderoy *et al.* 2020). Previous reports have demonstrated that mitophagy promotes NDV replication by blocking intrinsic apoptosis (Meng *et al.* 2014). However, the role of pexophagy in apoptosis remains unclear. Our hypothesis suggests a link between pexophagy and apoptosis, which needs to be further clarified.

5. Conclusion

This study uncovers a new mechanism whereby NDV-induced apoptosis is regulated *via* the suppression of *PEX* expression. Specifically, NDV infection initiates the degradation of PEX5, which in turn facilitates apoptosis by regulating Bcl-2. Thus, this study sheds light on the potential role of peroxisome in apoptosis regulation in response to virus infection.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

Not applicable.

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