

Original Article

Effect of human protein kinase R-like endoplasmic reticulum kinase gene on the apoptosis of LO2 hepatocytes under endoplasmic reticulum stress

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Abstract: Background: To evaluate the effects of recombinant lentiviral particles for human lentiviral vector carrying protein kinase R-like endoplasmic reticulum kinase (PERK) gene on endoplasmic reticulum (ER) stress-mediated apoptosis of human LO2 hepatocytes. Methods: 293T cells were co-transfected with recombinant lentiviral vector pWPT-GFP-PERK as well as packaging plasmids pMD2G and pSPAX2, giving a PERK-expressing lentivirus (LV-PERK). The supernatant was collected 48 h after transfection to infect LO2 hepatocytes. Infection efficiency was detected by observing GFP expression. The effects of LV-PERK on the apoptosis of LO2 cells under ER stress were assessed by flow cytometry. The expressions of apoptosis-related cleaved caspase-3 and CHOP proteins were detected by Western blotting. Results: Recombinant PERK lentivirus with titer of 4.2×10^8 efu/mL was constructed and packaged. Proportion of G1-phase cells in the tunicamycin (TM) + LV-PERK group was 53.49%, which was lower than those of TM (78.94%) and LV-GFP (65.73%) groups. The TM + LV-PERK group (32.45%) had significantly higher proportion of S-phase cells than those of TM (13.23%) and LV-GFP (17.79%) groups ($P < 0.05$). The apoptotic rate of the TM + LV-PERK group (26.55%) significantly exceeded those of TM (12.59%) and LV-GFP (11.43%) groups ($P < 0.05$). Western blotting results were consistent with those of flow cytometry. Conclusion: Under ER stress, LV-PERK promoted the proliferation and apoptosis of LO2 hepatocytes.

Keywords: Lentivirus, hepatocyte, protein kinase R-like endoplasmic reticulum kinase, gene modification

Introduction

Proteins are folded and modified as well as calcium ions are stored in the endoplasmic reticulum (ER). Protein kinase R-like endoplasmic reticulum kinase (PERK), as a type I transmembrane protein on ER, belongs to the upstream kinase family of eukaryotic initiation factor 2 α (eIF2 α) and has the activity of serine/threonine protein kinase [1]. ER stress is activated when considerable misfolded proteins accumulate in ER, so PERK, as an important signaling molecule, is further activated to inhibit protein synthesis through phosphorylation of eIF2 α . Meanwhile, the expression of C/EBP homologous protein (CHOP) gene is up-regulated through activating transcription factor 4 (ATF4), thereby inducing cell apoptosis [2]. Under ER stress, PERK gene plays crucial roles in the

apoptosis of INS-1 [3] and H4IIE cells [4], but the relationship between PERK gene and apoptosis of human hepatocytes under this stress has seldom been studied. In this study, recombinant lentiviral particles for human PERK gene were successfully constructed and packaged to evaluate the effects on human LO2 hepatocytes under ER stress. The findings pave the way for clarifying the biological functions of PERK gene, and provide theoretical evidence for unraveling the mechanisms for skeletal muscle injury and repair.

Materials and methods

Materials

Lentiviral expression vector pWPT-green fluorescent protein (GFP) as well as lentiviral pack-

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aging vectors pMD2G and pSPAX2 was purchased from Biovector Science Lab, Inc. HEK-293T and LO2 cells were preserved in our group. Lipofectamine 2000 was obtained from Life Technologies. TRIzol reagent, SYBR Real-time PCR Master Mix, exonuclease III, BamH I, Mlu I endonuclease, gel purification kit, DNA extraction kit and primers were bought from TaKaRa. Plasmid Miniprep Kit was purchased from Omega. DMEM and fetal bovine serum were provided by Hyclone. Cell culture dishes were obtained from NEST. ECL kit was bought from Beijing Keyushenlan Technology Co., Ltd. PVDF and NC membranes were purchased from Milipore. β -Actin antibody, PERK antibody, eIF2 α antibody and HRP-labeled goat anti-rabbit/goat anti-mouse antibodies were provided by Santa Cruz. RIPA protein lysate was bought from Shanghai Bogoo Biotechnology Co., Ltd.

Construction of PERK lentiviral vector

Primer design and synthesis: According to the gene sequence in the CDS region of human PERK in GenBank, primers for PERK amplification were designed and synthesized. Sense primer: tcgtgacgcGGATCCATGGAGCGGCCATCAGC; antisense primer: agcgctaggacgcgtATTGCTTGGCAAAGGGCTATGG (underlined parts: Bam HI and Mlu I endonuclease sites). PERK gene (3348 bp) was amplified by using eukaryotic expression vector pcDNA3.1(-)-PERK as the template.

PCR amplification of target gene and construction of PERK-containing lentivirus: PERK gene was amplified in a 100 μ L PCR system, comprising 50 μ L of 2 \times Taq Master Mix, 2.5 μ L of upstream and downstream primers each, 5 μ L of template and 40 μ L of sterile double-distilled water. Reaction conditions: Pre-denaturation at 94 $^{\circ}$ C for 5 min, denaturation at 94 $^{\circ}$ C for 30 s, annealing at 64 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 3 min 45 s, 35 cycles in total; re-extension at 72 $^{\circ}$ C for 5 min. The PCR products were stored at 4 $^{\circ}$ C and separated by 1% agarose gel electrophoresis. Lentiviral vector pWPT-GFP was subjected to double digestion with restriction endonucleases Bam HI and Mlu I and ligated with the purified PCR products through ligation-independent cloning, constructing vector pWPT-GFP-PERK. The product was transformed into DH5 α competent cells and shaken at 37 $^{\circ}$ C overnight. Plasmid DNA extracted with Plasmid Miniprep Kit, digested, PCR-identified, and sequenced.

Package of recombinant lentivirus and transfection of target cells

293T cells in the logarithmic growth phase were inoculated onto two 6 cm culture dishes 1 d before transfection. On the second day, pWPT-GFP and pWPT-GFP-PERK were mixed with packaging plasmids pSPAX2 and pMD2G respectively to transfect 293T cells according to the instructions of LipofectamineTM 2000. The doses of the three plasmids were 12.5 μ g, 7 μ g and 3.5 μ g respectively. The culture medium was refreshed 5 days later, and the supernatant was collected at 48 h and 72 h, filtered through a 0.45 μ m filter and stored at -80 $^{\circ}$ C.

Determination of viral titer

293T cells were inoculated into 24-well plates 1 d before infection and added diluted viral supernatant (200 μ L) with the double dilution method. Two replicate wells were set for each dilution factor. The cells were cultured in an incubator overnight, and the culture medium was replaced with DMEM-complete on the second day. The cells emitting green fluorescence were counted 48 h later under a fluorescence microscope. One cell that emits green fluorescence is an expression forming unit (efu). Viral titer (efu/mL) and multiplicity of infection (MOI) can be calculated according to the following equations: Viral titer = Number of cells emitting green fluorescence \times dilution factor/0.25. MOI = Viral titer \times volume of added virus/number of infected cells.

Detection of PERK mRNA expressions in LV-PERK-infected LO2 cells by RT-PCR

LO2 cells reaching 90% confluence in 6 cm culture dishes were collected and divided into a blank control group, an LV-GFP-infected group and an LV-PERK-infected group. Total RNA was extracted by TRIzol, reverse-transcribed into cDNA with PrimeScriptTM RT Reagent Kit, amplified by PCR and revolved by 1% agarose gel electrophoresis. Primer sequences of PERK: Upstream: 5'-AGCAC-TCAGATGGAGAGAGTCAG-3', downstream: 5'-GCTATGGGAGTTGTTGGACTGT-3', product size: 260 bp. Primer sequences of β -actin: Upstream: 5'-GACCCAGATCATGTTTGAGACC-3', downstream: 5'-ATCTCCTTCTGCATCCTGTGC-3', product size: 550 bp. Reaction system of RT-PCR: 5 μ L of 2 \times Taq Master Mix, 0.4 μ L of upstream and downstream primers each, 2 μ L of tem-

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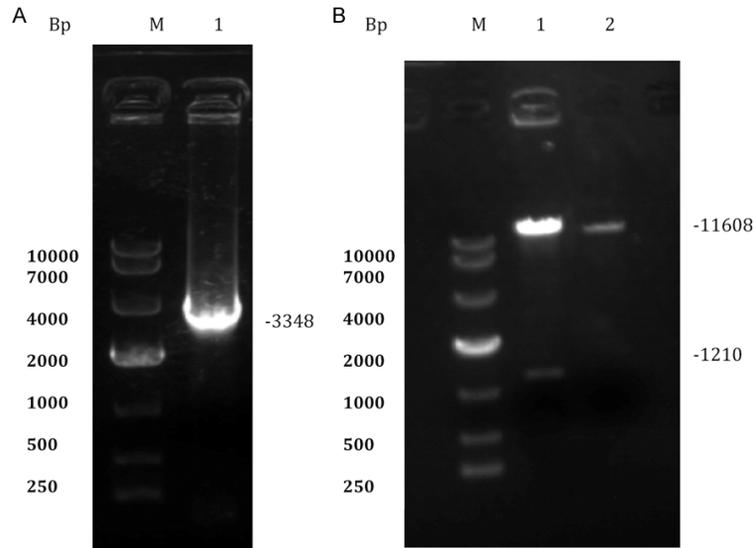


Figure 1. PCR results of PERK gene and digestion results of lentiviral vector pWPT-GFP-PERK. A: PCR results of PERK (M: DL10000; 1: pcDNA3.1(-)-PERK); B: BamH I digestion results of pWPT-GFP-PERK (M: DL10000; 1: pWPT-GFP-PERK; 2: pWPT-GFP).

plate and 2.2 μ L of sterile double-distilled water. Reaction conditions: Pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, 30 cycles in total; re-extension at 72°C for 10 min. The PCR products were stored at 4°C and separated by 1% agarose gel electrophoresis.

Detection of PERK protein expressions in LV-PERK-infected LO2 cells by Western blotting

LO2 cells reaching 90% confluence in 6 cm culture dishes were collected and divided into a blank control group, an LV-GFP-infected group and an LV-PERK-infected group. The cells were washed three times with pre-cooled PBS, added 200 μ L of protein lysate, slowly scraped off into 1.5 ml EP tubes, lysed on ice for 40 min, and centrifuged at 12000 \times g and 4°C for 15 min. The supernatant was collected into new EP tubes, added 4 \times SDS loading buffer, subpackaged and stored at -80°C. The three proteins (20 μ L) were separated by 10% SDS-PAGE, electronically transferred to a PVDF membrane for 1 h, blocked with 5% skim milk for 1 h, incubated overnight with mouse β -actin antibody (1:1500) and rabbit PERK antibody at 4°C, incubated with horseradish peroxidase-labeled secondary antibodies (goat anti-mouse, 1:5000; goat anti-

rabbit, 1:5000), and image-developed by ECL reagent.

Detection of LO2 cell cycle and apoptosis by flow cytometry

Detection of cell cycle: LO2 cells in the logarithmic growth phase were inoculated into 6-well plates and added pWPT-GFP-PERK or pWPT-GFP at 80% confluence. Meanwhile, a normal control (NC) group was set. Two replicate wells were set for each group, and each experiment was performed in triplicate. Tunicamycin (TM) was added in one experimental group, and the other group was treated for 24 h with 4 μ g/mL TM 72 h after infection. All cells were then fixed with 1.5 mL of 70%

ethanol for 24 h, added PI at the same volume, left still at 4°C for 20-30 min, filtered through nylon membrane, and detected by flow cytometry in Institute of Life Sciences, Chongqing Medical University.

Detection of cell apoptosis: LO2 cells in the logarithmic growth phase were inoculated into 6-well plates and added pWPT-GFP-PERK or pWPT-GFP at 80% confluence. Meanwhile, an NC group was set. Two replicate wells were set for each group, and each experiment was performed in triplicate. TM was added in one experimental group, and the other group was treated for 24 h with 4 μ g/mL TM 72 h after infection. All cells were then washed three times with PBS, added PBS at the same volume, and detected by flow cytometry with the Annexin PE method in Institute of Life Sciences, Chongqing Medical University.

Detection of apoptosis-related proteins in LO2 cells

LO2 cells in the logarithmic growth phase were inoculated into 6-well plates and added pWPT-GFP-PERK or pWPT-GFP at 80% confluence. Meanwhile, an NC group was set. The cells were treated for 24 h with 4 μ g/mL TM 72 h after infection, and total proteins were extracted. The expressions of PERK protein, PERK phosphorylation-related protein eIF2 α as well

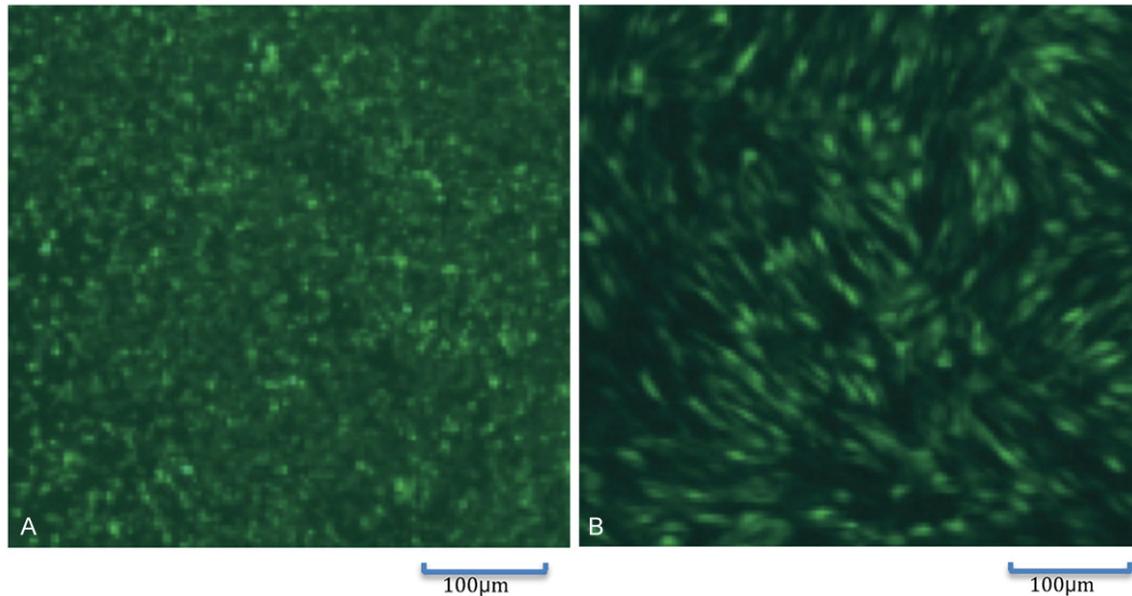


Figure 2. Lentiviral packaging and infection of LO2 cells. A: Lentiviral packaging in 293T cells; B: Lentivirus LV-PERK infected LO2 cells.

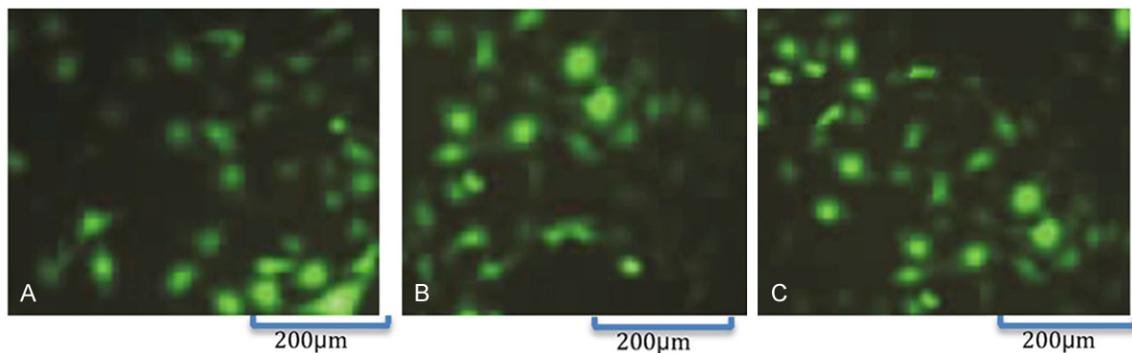


Figure 3. Detection of viral titers in LO2 cells. A: MOI = 20; B: MOI = 40; C: MOI = 100.

as apoptosis-related cleaved caspase-3 and CHOP proteins were detected by Western blotting.

Statistical analysis

All data were analyzed by SPSS15.0. Inter-group comparisons were performed with analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Construction of lentiviral vector

As expected, target gene PERK sized 3348 bp was amplified by using pcDNA3.1(-)-PERK as the

template (**Figure 1A**). PERK and pWPT-GFP were subjected to double digestion with BamH I and Mlu I, purified, ligated, transformed, plasmid-extracted and identified. Only BamH I digestion was conducted because PERK gene contained an endonuclease site for BamH I. As shown in **Figure 2B**, there are two bands at 1210 bp and 11608 bp, while the control plasmid only presents one band. Therefore, lentiviral expression vector pWPT-GFP-PERK was successfully constructed.

Lentiviral packaging and infection of LO2 cells

293T cells were observed under the fluorescence microscope 48 h after co-transfection with pWPT-GFP-PERK and pMD2G, pSPAX2,

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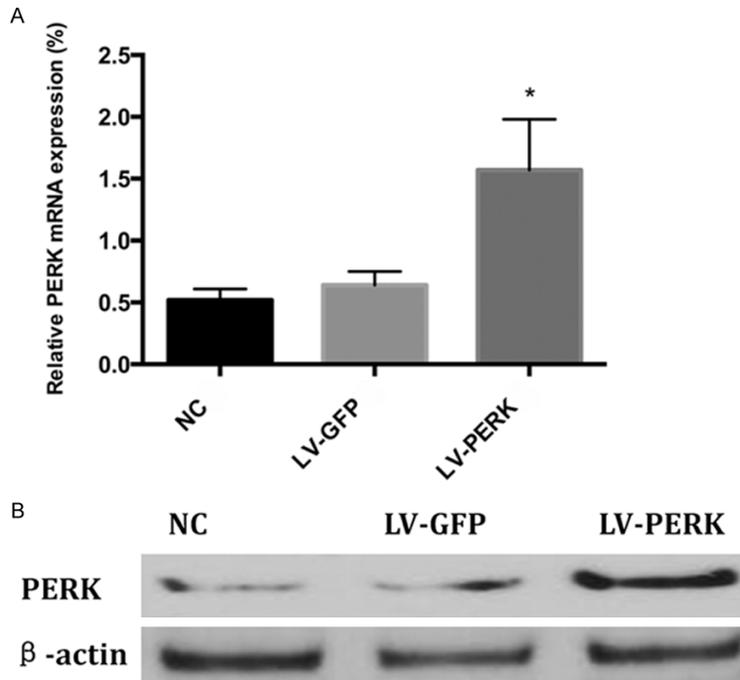


Figure 4. Expressions of PERK mRNA and protein in LO2 cells. A: Expression of PERK mRNA in LO2 cells detected by RT-PCR (vs. NC group/LV-GFP group, * $P < 0.05$); B: Expression of PERK protein in LO2 cells detected by Western blotting.

showing green fluorescence. In the meantime, the cells stopped growing, suggesting lentiviral packaging was successful (Figure 2A). The packaged LV-PERK was filtered through a 0.45 μm filter to infect LO2 cells that emitted green fluorescence 72 h later. The proportion of cells expressing GFP was higher than 90% (Figure 2B).

Viral titer

The titer of LV-PERK was, as detected by the double dilution method, 4.2×10^8 efu/ml. LO2 cells were infected with LV-PERK at MOI 20 (Figure 3A), 40 (Figure 3B) and 100 (Figure 3C). After 72 h, the GFP expression level at MOI 40 exceeded 40%, but that at MOI 100 decreased, indicating that the optimum MOI was 40.

PERK mRNA and protein expressions in LO2 cells

RT-PCR results showed that the PERK mRNA expression levels of the NC and LV-GFP groups significantly lower than that of the LV-PERK group (Figure 4A) ($P < 0.05$). Western blotting showed that significantly more PERK protein

was expressed in the LV-PERK group than those in the NC and LV-GFP groups 72 h after infection (Figure 4B).

Effects of LV-PERK on LO2 cell cycle

Without TM treatment, the cell cycles of all groups barely changed (Figure 5A). Under TM-induced ER stress, however, the LV-PERK group had significantly lower (53.49% vs. 78.94% and 65.73%) and significantly higher (32.45% vs. 13.23% and 17.79%) proportions of G1-phase and S-phase cells respectively than those of the TM and LV-GFP groups ($P < 0.05$) (Figure 5B). Thus, PERK promoted the proliferation of LO2 cells under ER stress.

Effects of LV-PERK on LO2 cell apoptosis

Without TM treatment, the apoptotic rate of LO2 cells was only 4.12% (Figure 6). The TM + LV-PERK group had significantly higher apoptotic rate than those of the LV-PERK, TM and TM + LV-GFP groups ($P < 0.05$). Accordingly, PERK accelerated the apoptosis of LO2 cells under ER stress.

Effects of PERK protein expression and LV-PERK on LO2 cell apoptosis under ER stress

The expressions of PERK protein as well as apoptosis-related cleaved caspase-3 and CHOP proteins under ER stress were detected by Western blotting. Compared with the NC group (Figure 7), endogenous PERK expression slightly increased in TM-treated LO2 cells under ER stress. Such expression was evidently increased in LO2 cells infected with LV-PERK than those in the uninfected cells. Besides, significantly more PERK phosphorylation-related eIF2 α , cleaved caspase-3 and CHOP proteins were expressed in the LV-PERK group under ER stress than those in the TM and TM + LV-GFP groups. Hence, LV-PERK facilitated the apoptosis of LO2 cells under ER stress.

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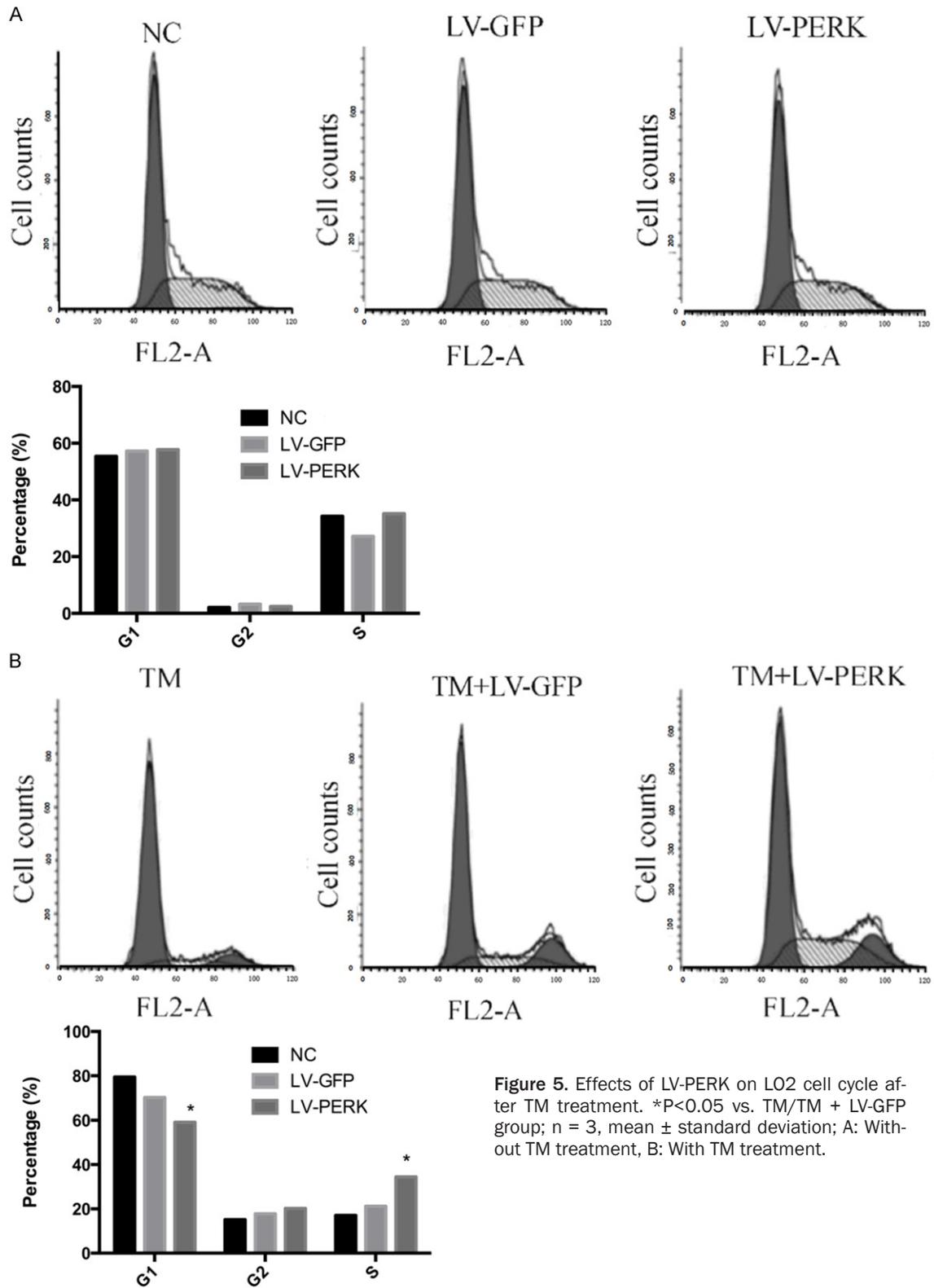


Figure 5. Effects of LV-PERK on LO2 cell cycle after TM treatment. *P<0.05 vs. TM/TM + LV-GFP group; n = 3, mean ± standard deviation; A: Without TM treatment, B: With TM treatment.

Discussion

As a crucial organelle in cells, ER is the main place for protein modification, folding and

processing in eukaryotic cells as well as for calcium ion storage. ER stress is induced upon accumulation of unfolded or misfolded proteins in ER cavity or disruption of intracellu-

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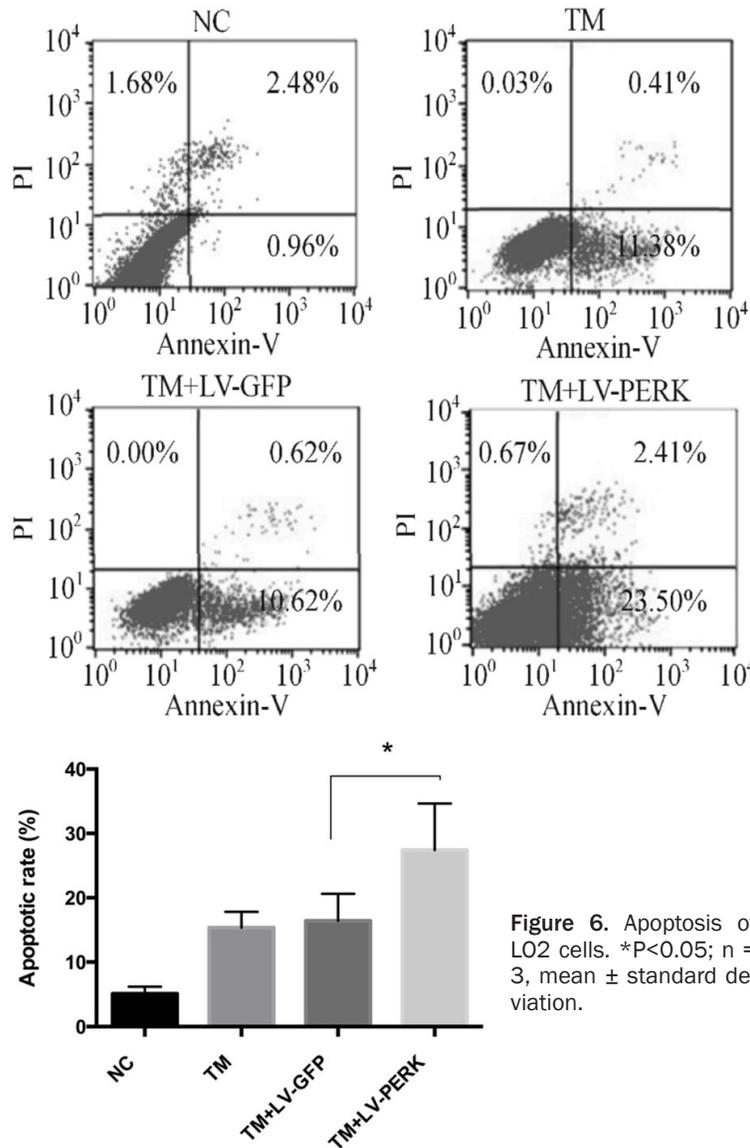


Figure 6. Apoptosis of LO2 cells. *P<0.05; n = 3, mean ± standard deviation.

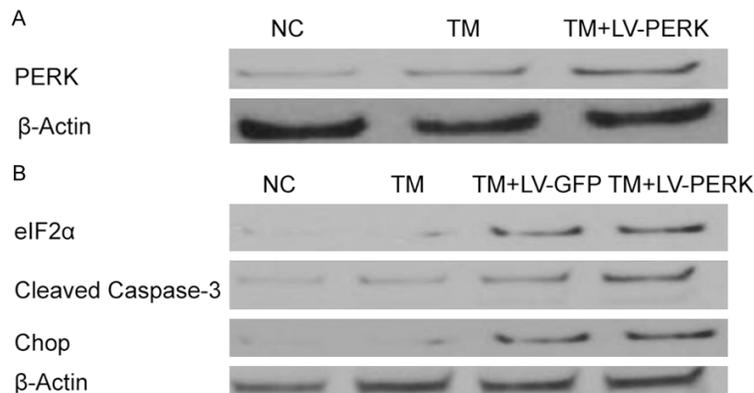


Figure 7. Expressions of PERK protein and ER stress-mediated apoptosis-related proteins in LO2 cells. A: Endogenous expression of PERK protein in LO2 cells under ER stress; B: Expression of eIF2α and ER stress-mediated apoptosis-related proteins in LO2 cells.

lar calcium homeostasis [5]. PERK is a type I transmembrane protein on ER and has the activity of serine/threonine protein kinase [6, 7], and the PERK pathway is activated under ER stress. Under this circumstance, PERK phosphorylates eIF2α and then inhibits the exchange between GDP and GTP in translation initiation complexes, thus suppressing the synthesis and translation of proteins [8]. During ER stress-mediated apoptosis, PERK temporarily terminated protein synthesis and activated ATF4 to up-regulate the expression of CHOP that inhibits Bcl-2 expression, finally leading to cell apoptosis through caspase-3 [9-11].

ER stress mediates cell apoptosis [12], and the apoptosis of hepatocytes is implicated in many types of liver diseases. Hepatitis B is accompanied by hepatocyte apoptosis which plays key roles in disease onset and progression. Viral hepatitis involves many factors that can trigger apoptosis, including immune effector cells and cytokines during infection, post-infectious cellular DNA damage and cell cycle disorders [13]. Hepatocyte apoptosis is mainly manifested as spotty necrosis and piecemeal necrosis in liver lobules upon viral hepatitis. The apoptosis pathways of hepatocytes basically resemble those of receptor families. For example, the ICE family plays central roles in cell apoptosis, being responsible for the common pathways that apoptotic signals share. In addition, Rb, nm23 and bax genes prevent hepatocarcinoma by suppressing hepatocyte growth or promoting apoptosis.

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In this study, we established a typical ER stress model by infecting LO2 hepatocytes with lentivirus LV-PERK and by continuously treating them with TM [14]. The effects of ER-related PERK gene on ER stress-induced apoptosis of LO2 cells were assessed by detecting apoptosis-related CHOP and caspase-3 through flow cytometry and Western blotting. Under continuous ER stress, the proliferative rate of the TM + LV-PERK group surpassed that of the TM group, and the former had higher proportion of S-phase cells and lower proportion of G1-phase ones than those of the latter. Therefore, PERK promoted the proliferation of LO2 cells under ER stress by facilitating the growth from G1 phase to S phase. Since the TM group had significantly higher apoptotic rate than that of the NC group and the rate of the TM + LV-PERK group exceeded that of the TM group, PERK was conducive to the apoptosis of LO2 cells under continuous ER stress. Similarly, Western blotting showed that the expressions of cleaved caspase-3 and CHOP proteins in the TM and TM + LV-GFP groups were significantly up-regulated, and that the TM + LV-PERK group had significantly higher apoptotic rate than that of the TM group. Thus, PERK accelerated LO2 cell apoptosis under ER stress, probably because activation of PERK further activated the downstream apoptosis pathways. It has previously been reported in osteoblasts that the PERK-eIF2 α -ATF4 pathway participated in cell differentiation under the mediation of ER stress [15]. Moreover, the PERK-ATF4-ATF4-CHOP pathway predominantly controlled ER stress-induced apoptosis of mouse MIN6 and INS-1 cells [4, 16]. Although the pathogenesis of many liver diseases are closely associated with hepatocyte apoptosis, whether PERK-related signaling pathways are involved remains unclear. We are going to apply this expression vector to studies on the roles of PERK gene and related signaling pathways in ER stress-induced hepatocyte apoptosis.

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Disclosure of conflict of interest

None.

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