ANTICANCER RESEARCH *40*: 4491-4504 (2020)
doi:10.21873/anticanres.14454

Peroxiredoxin II Inhibits Alcohol-induced Apoptosis in L02 Hepatocytes Through AKT/β-Catenin Signaling Pathway

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Abstract. *Background: Peroxiredoxin II (PRDX2) performs unique roles in cells. It can reduce peroxides through cysteine residues, and helps prevent the effects of oxidative stress on cells. It is closely related to the occurrence and development of various diseases, especially alcoholic liver injury and even liver cancer. The metabolism of alcohol in hepatocytes leads to the increase in the levels of reactive oxygen species (ROS), oxidative stress, injury, and apoptosis. Therefore, this study focused on the investigating the protection conferred by PRDX2 against alcohol-induced apoptosis of hepatocytes. Materials and Methods: PRDX2 inhibition of alcohol-induced apoptosis in L02 hepatocytes was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, fluorescence microscopy, flow cytometry, western blotting and hematoxylin and eosin staining. Results: The results showed that the levels of reactive oxygen species, protein kinase B, β-catenin, B-cell lymphoma-2 (BCL2), BCL-XL, BCL2-associated X, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase in PRDX2-silenced cells were increased significantly after the treatment of cells with ethanol. Similar results were obtained in an in vivo Prdx2-knockout mouse model of alcoholic liver injury. Therefore, PRDX2 may regulate the phosphorylation of the AKT signal protein by eliminating reactive oxygen species from cells, and it inhibits the downstream mitochondria-dependent apoptosis pathway, and, thereby, the apoptosis of cells. Conclusion: Thus, PRDX2 may be a potential molecular target for the prevention and treatment of alcoholic liver injury.*

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Key Words: Peroxiredoxin II, PRDX2; ROS, liver injury, cell apoptosis.

The relationship between alcohol and health has always been a research topic of great concern. Alcohol intake is associated with injury to organs, cancer, cardiovascular diseases, cirrhosis, and other diseases, especially liver damage, and can, therefore, be a direct or indirect cause of death (1). Alcoholic liver disease has become one of the main diseases threatening human health because of its complicated pathogenesis, subjective dependence, and social issues (2). In 2010, nearly 50% of the world's population was drinking alcohol in some or other, and more than one million deaths worldwide were attributed to cirrhosis, with 47.9% of those who died had a long history of alcohol abuse (3). In 2012, 5.9% of deaths globally were related to alcohol abuse. Among long-term alcoholics, 80-90% suffer from alcoholic fatty liver, 20-40% develop liver fibrosis, disease in 8-20% progresses to cirrhosis and ascites, and 3-10% develop hepatocellular carcinoma (4).

Alcoholic liver disease begins with steatosis, which is characterized by the rise of triglycerides in the liver. The histological features of advanced alcoholic liver disease are necrosis and apoptosis of hepatocytes, and are important features of alcoholic liver disease in clinical practice (5). Early studies on the pathogenesis of alcoholic liver disease mainly

focused on metabolism-related oxidative stress and glutathione consumption, abnormalities in methionine metabolism, malnutrition, and the production of endotoxin to activate Kupffer cells (6). With the development of research, reactive oxygen species (ROS) have been found to play important roles in alcoholic liver disease (7). Accumulation of ROS can cause oxidative stress, endoplasmic reticulum stress, and steatosis of hepatocytes, which lead to hepatocyte damage and eventually to various liver diseases (8). The metabolism of alcohol is an important source of ROS. In the human body, alcohol is mainly metabolized by alcohol dehydrogenase and the microsomal alcohol oxidation system in the liver to acetaldehyde; in this process, much active oxygen is produced. One of the main causes of alcoholic liver disease is hepatitis caused by oxidative stress and lipopolysaccharides (9). Therefore, for the protection of hepatocytes, it is very important to maintain ROS homeostasis in these cells.

The liver, which is the detoxification organ in the human body, contains rich antioxidant systems, including peroxiredoxins (PRDXs), superoxide dismutase, glutathione peroxidase and catalase that are involved in the removal of ROS and in the protection of liver during the metabolism of alcohol (10). Peroxiredoxins belong to the superfamily of mercaptan-dependent peroxidases, which were discovered in yeast (11). Unlike other antioxidant enzymes, PRDXs do not contain redox cofactors, such as Fe^{2+} or ferroglobin. Instead, they use thioredoxin as an intermediate H^+ donor to neutralize peroxides. PRDXs are abundant in cytoplasm, accounting for 1% of the total soluble protein (12). They play an important role in many biological processes, such as in apoptosis and the proliferation of cells (13). It has been shown that many PRDXs involved in alcohol metabolism, such as PRDX3 and PRDX5, are peroxidized, resulting in redox imbalance in the mitochondria of alcohol-treated alveolar macrophages (14). We previously showed that PRDX might play a protective role in alcohol-induced liver injury. This study investigated the protective effect of PRDX2 on alcohol-induced injury to hepatocytes by constructing a stable *PRDX2*-silenced L02 human normal hepatocyte line *in vitro*, and deciphered the theoretical basis underlying the mechanism through which PRDX2 protects against hepatocyte injury for further exploration.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Solarbio (Solarbio Life Sciences, Beijing, PR China). The antibodies used were from Bioss (Bioss Biology, Beijing, PR China) and Santa (Santa Cruz Biotechnology, Santa Cruz CA, USA). The ethanol was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Cell culture plates were purchased from NEST (NEST Biotechnology, Wuxi, Jiangsu, PR China), and the fluorescence microscope was from Leica (Wetzlar, Germany).

Cell culture. The cryopreserved L02 cells shipped by the vendor on dry ice were frozen in liquid nitrogen for 24 h upon receipt. The frozen cells were rapidly thawed, almost completely, by placing the tubes in a water bath at 37˚C. They were added to 5 ml of culture medium (DMEM supplemented with 20% FBS and 1% penicillin-streptomycin solution) in a centrifuge tube and pelleted by centrifugation at 92 × *g* for 3 min. The cells were thoroughly resuspended in the culture medium, plated on culture dishes (10-cm diameter), and incubated in an incubator at 37˚C with 5% CO_2. In some experiments, cells were pretreated with 20 ng/ml fibroblast growth factor 2 (FGF2), an AKT agonist for 2 h. These cells were then treated with 400 mM ethanol for 12 h, and harvested.

Generation and screening of lentivirus-transfected human normal hepatocyte lines. The mRNA sequence of human *PRDX2* gene (serial number NM_005809) was searched in the National Center of Biotechnology Information database. An oligonucleotide (5'-cgcuugugaguaguacuacuacuacugu-3') specific for this gene and green fluorescent protein *(GFP)* gene as marker was used for generating the lentivirus vector construct for silencing of this gene using short hairpin RNA (sh*PRDX2*). L02 cells were subcultured to second passage, and 3×10^5 cells per well were seeded in 6-well plates. The cells were divided into blank, mock, and sh*PRDX2* groups. Based on the multiplicity of infection, the cells were infected with appropriate volumes of the virus carrying the constructs after 12 h of subculture, and after 24 h, the infection was stopped, and the cells were subcultured in 10-cm culture dishes. The transfected cells were screened in the presence of 2 μg/ml purinomycin. When L02 cell lines cultured in normal medium were suspended at the same density, cells successfully transfected with lentivirus were screened. Three groups cells (blank, mock, and sh*PRDX2*) were subcultured in two 3.5-cm plates for each group, with each plate containing 2×10^5 cells. After 12 h, the cells were harvested and the transfection rate was determined by flow cytometry. After 24 h, the cells in the second set of plates were harvested and analyzed for *PRDX2* expression by immunoblotting.

Evaluation of the cytotoxicity of ethanol using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Mock-transfected and sh*PRDX2*-transfected cells were evenly seeded in 96-well plates at a density of 5,000 cells per well and cultured for 24 h in 10% FBS-supplemented DMEM containing 0, 100, 200, 300, 400, 500, or 600 mM ethanol.

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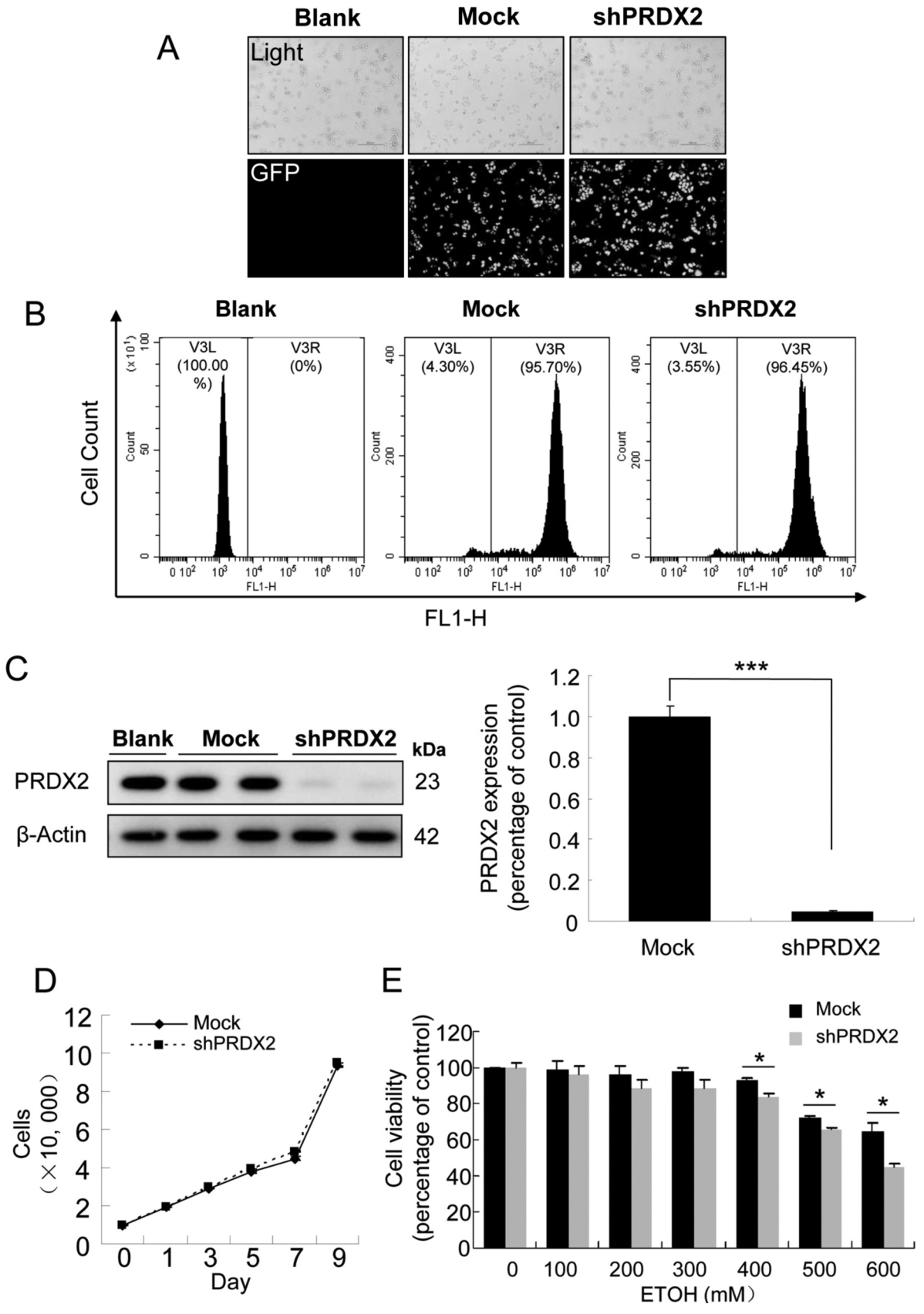
Figure 1. *Establishment of stable peroxiredoxin II gene (PRDX2)-silenced L02 hepatocyte line. A: Green fluorescent protein (GFP) expression in blank (L02 cells), mock (L02 cells transfected with empty lentivirus particles), and L02 cells transfected with lentivirus particle containing PRDX2 knockdown sequence (shPRDX2) cells as observed under a fluorescence microscope. B: Flow cytometric analysis of cells in the three groups. C: Expression of PRDX2 in the three groups as assessed by immunoblotting. D: Growth curves of cells in mock and shPRDX2 groups prepared using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. E: Toxicity of ethanol at different concentrations in the cells of mock and shPRDX2 groups as assessed by MTT assay. Quantitative data are presented as means±SEM. *Significantly different at p<0.05.*


A
Blank
Mock
shPRDX2
Light
GFP
B
Blank
Mock
shPRDX2
V3L (100.00 %)
V3R (0%)
V3L (4.30%)
V3R (95.70%)
V3L (3.55%)
V3R (96.45%)
Cell Count
FL1-H
C
Blank
Mock
shPRDX2
kDa
PRDX2
23
β-Actin
42
PRDX2 expression (percentage of control)
***
Mock
shPRDX2
D
Mock
shPRDX2
Cells (×10, 000)
Day
E
Mock
shPRDX2
Cell viability (percentage of control)
ETOH (mM)

Thereafter, 10 μl MTT (Sigma-Aldrich) was added to each well and the plates were allowed to stand for 4 h. The formazan crystals thus formed were dissolved by adding 100 μl dimethylsulfoxide to each well and incubating the plates in a CO_2 incubator at 37˚C for 10 min. The absorbance was read at 490 nm using a microplate reader. Quantitative analysis was carried out through SPSS Statistics.

Detection of apoptosis by fluorescence microscopy. The mock and sh*PRDX2* cells were seeded in 6-well plates at a density of 2×10^5 cells per well. They were divided into three groups according to ethanol treatment: control group, 12-h treatment group, and 24-h treatment group. The cells in the treatment groups were treated with 400 mM alcohol for the time designated. The dead cells were then washed off using phosphate-buffered saline (PBS), and 500 μl each of phycoerythrin-conjugated annexin V (Solarbio Life Sciences) and dye-binding buffer were added to each well of the 6-well plate. After incubating at room temperature for 15 min, the cells were observed under a fluorescence microscope at 400× and 200× magnification. Quantitative analysis was carried out through Image J and SPSS Statistics.

Detection of apoptosis by flow cytometry. A total of 3×10^5 mock and sh*PRDX2* transfected cells were evenly seeded in each well of 6-well plates. They were then divided into two groups, namely the control and the group treated with 400 mM ethanol for 12 hours. After 24 h of culture in DMEM containing 10% FBS, the culture medium was replaced with DMEM containing 1% FBS. After 12 h of further culture, the medium was removed, and the adherent cells were harvested after digestion with trypsin (without EDTA). The cell suspension was centrifuged at 825 × *g* for 4 min. The cell pellet was washed once with 500 μl PBS, which was followed by centrifugation at 825 × *g* for 4 min. The supernatant was discarded and the cell pellet from each sample was resuspended in 200 μl binding solution and 1 μl annexin V-APC solution (Solarbio Life Sciences), and incubated in the dark for 15 min at room temperature. These cells were transferred to flow tubes, and mixed with 300 μl PBS. Apoptosis was detected using a Beckmann flow cytometer (Miami, FL, USA). Quantitative analysis was carried out through SPSS Statistics.

Cell-cycle detection. A total of 3×10^5 mock and sh*PRXD2*-transfected cells per well were evenly seeded in 6-well plates and divided into three groups according to the time for which they were treated with ethanol: Control group, 6-h treatment group, and 12-h treatment group. After 24 h of culture in DMEM containing 10% FBS, the culture medium was replaced with DMEM containing 1% FBS and the cells were treated with 400 mM ethanol for the time designated. After a further culture for 6 h, the cells were dislodged by trypsinization and pelleted by centrifugation at 825 × *g* for 4 min. The cells were washed with 500 μl PBS once, and pelleted again by centrifugation at 825 × *g* or 4 min. These cells were then added to 1 ml pre-cooled ethanol in 2 ml centrifuge tubes for each treatment and mixed by gentle vortexing at 4˚C. The tubes were allowed to stand for 20 min at 4˚C in a refrigerator. The cells were then pelleted by centrifugation at 825 × *g* for 4 min, the pellet was washed once with 500 μl PBS, and centrifuged again at 825 × *g* for 4 min. The supernatant was removed and the cells were resuspended in 500 μl PBS for cell-cycle analysis using a Beckman Coulter flow cytometer.

Detection of ROS by fluorescence microscopy. A total of 3×10^5 mock and sh*PRDX2* cells per well were evenly seeded in 6-well plates. They were divided into three groups according to the time for which they were treated with 400 mM ethanol as described above. Dead cells were washed off with PBS after the treatment. Thereafter, 500 μl dihydroethidium dye was added to each well, and the plate was incubated for 15 min at 37˚C in a CO_2 incubator. The supernatant was then discarded and 1 ml PBS was added to each well. The cells were visualized under a fluorescence microscope and photographed. The proportion of fluorescing cells relative to the total number of cells in the same field of vision was recorded.

Detection of protein expression by immunoblotting. The mock and sh*PRDX2*-transfected cells were harvested and lysed for 30 min. The homogenate thus obtained was centrifuged at 13201 × *g* for 20 min at 4˚C and the supernatant containing the proteins was collected. The protein concentration was determined using the Bradford method. Equal amounts (25 μg/ml) of protein samples were mixed with gel loading buffer, heated in a boiling water bath for 5 min and placed on ice, and were resolved by separated discontinuously on 12% sodium dodecyl sulfate-polyacrylamide gels electrophoresis. The proteins were transferred from the gel onto the nitrocellulose membrane. The membrane was blocked with 5% skimmed milk for 1 h and then washed for 30 min with 1X 10 mM Tris HCl (pH 7.5), 150 mM NaCl and 0.2% Tween-20 (TBST). The membrane was then incubated in primary antibody at room temperature for 1 h, washed with 1X TBST for 30 min, and then incubated with 5% skimmed milk for 10 min. It was then incubated in secondary antibody at room temperature for 1 h, and subsequently washed with 1X TBST for 30 min. The blot was developed using ECL, and the image was captured and analyzed using an imaging system. The different antibodies used in this study were as follows: protein kinase B (AKT), β-catenin, B-cell lymphoma-2 (BCL2), BCL-XL, BCL2-associated X (BAX), BCL2 associated agonist of cell death (BAD), cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP), caspase-3, phosphorylated (p) AKT1/2/3, AKT1/2/3, phosphorylated nuclear factor kappa-B protein 65 (p-NF-κB P65, cyclin D1, CDK4, heme oxygenase 1 (HO1), PARP1, PRDX2 (all Santa Cruz Biotechnology); p-β-catenin, β-catenin, cytochrome P450 2E1 (CYP2E1), β-actin, α-tubulin, FGF2 (all Bioss Biology).

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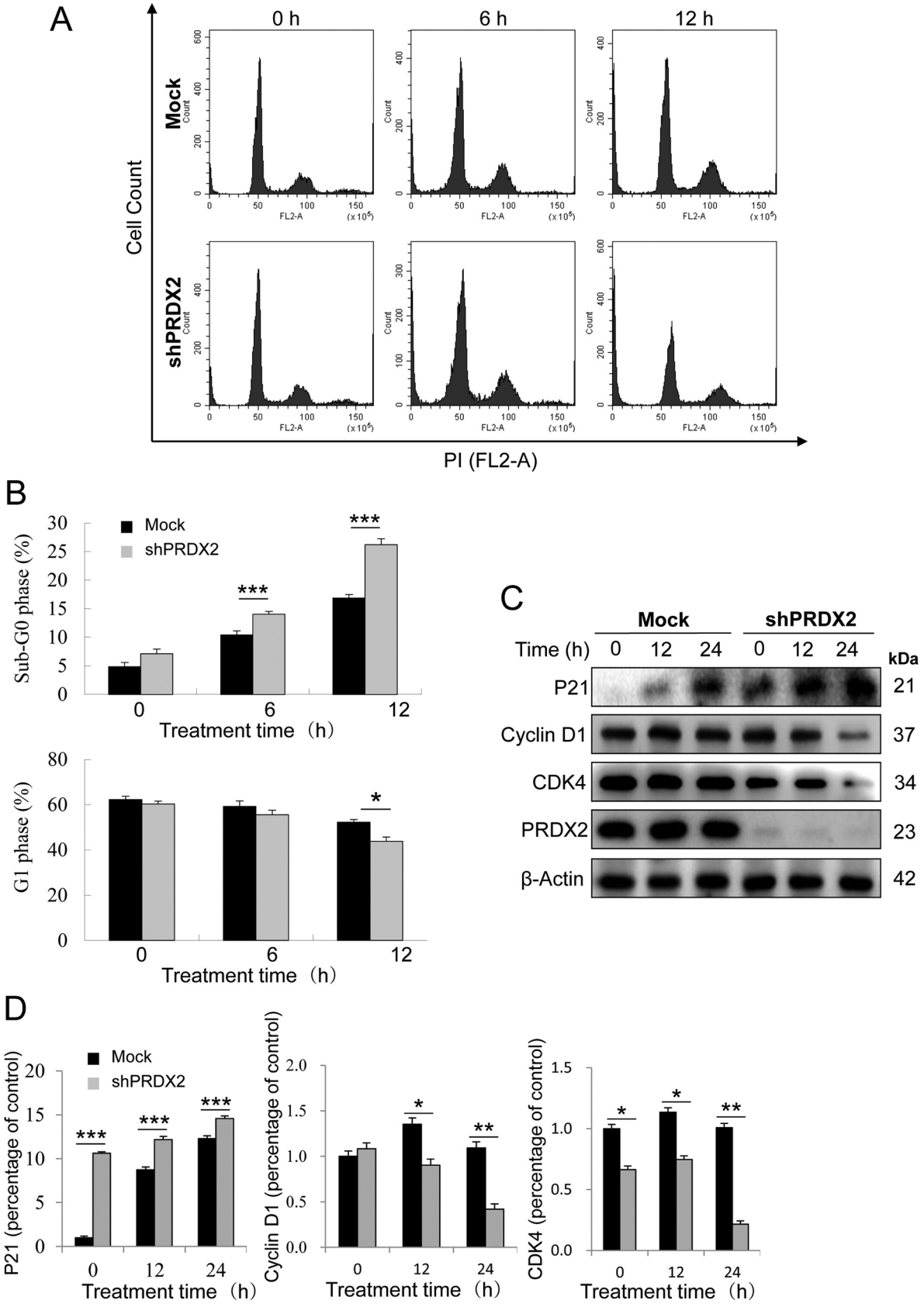
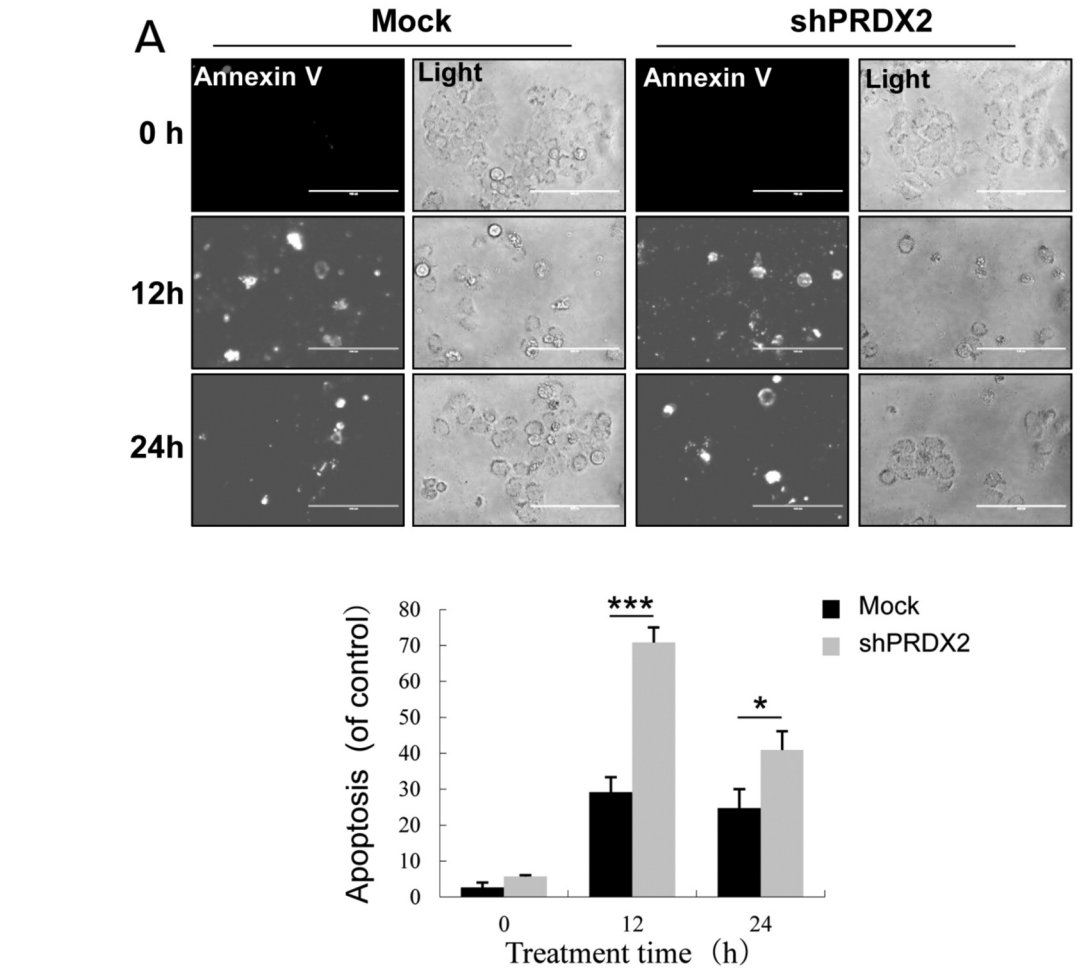
Figure 2. *Ethanol treatment alters the cell cycle in peroxiredoxin II gene-silenced L02 (shPRDX2) L02 hepatocytes, and induces cell-cycle arrest at G_1 phase. A: Cell-cycle distribution of cells in mock and shPRDX2 L02 hepatocyte lines treated with 400 mM ethanol for different durations, as detected using flow cytometry. B: Percentages of cells in sub-G_0 and G_1 phases of cell cycle in the groups treated with 400 mM ethanol for different durations. C: Effect of the treatment of cells in the two groups with 400 mM ethanol on the expression of different proteins. D: Quantification of the results presented in C. Quantitative data are presented as means±SEM for three independent experiments. Significantly different at *p<0.05, **p<0.01, ***p<0.001.*


A
0 h
6 h
12 h
Mock
shPRDX2
Cell Count
PI (FL2-A)
B
Mock
shPRDX2
Sub-G0 phase (%)
Treatment time (h)
G1 phase (%)
C
Mock
shPRDX2
Time (h)
kDa
P21
Cyclin D1
CDK4
PRDX2
β-Actin
D
P21 (percentage of control)
Cyclin D1 (percentage of control)
CDK4 (percentage of control)
Treatment time (h)



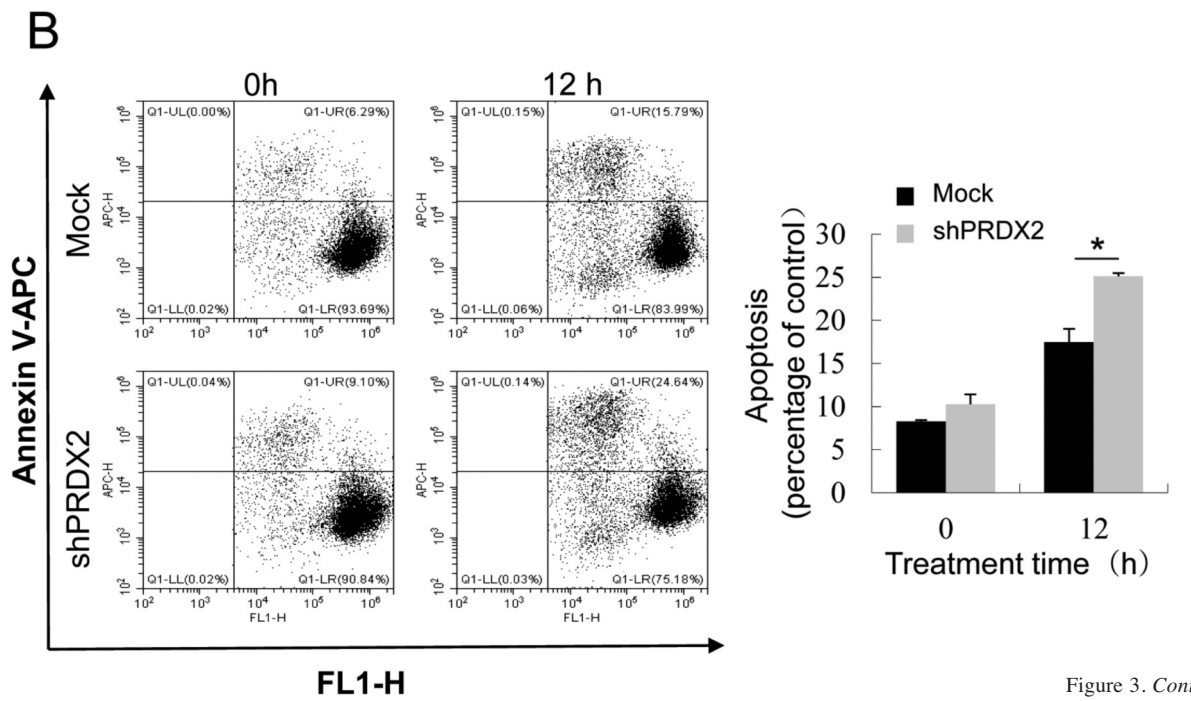


Figure 3. *Continued*

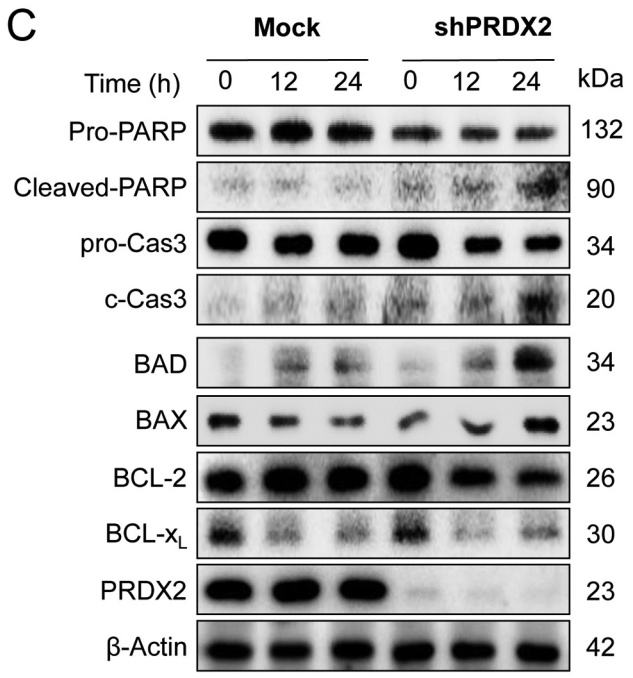


Figure 3. *Ethanol activates mitochondria-dependent apoptosis in peroxiredoxin II gene-silenced L02 (shPRDX2) L02 hepatocytes. A: Apoptosis was detected by immunofluorescence of annexin–phycoerythrin-stained mock and shPRDX2 L02 cells treated with 400 mM ethanol. The bar graph shows the fold-increase in the percentage of apoptotic cells relative to the control. B: Flow cytometric detection of apoptosis. The bar graph shows the rate of apoptosis in the two groups treated with ethanol for 12 h. C: Expression of procaspase-3 (proCas3), cleared caspase 3 (c-Cas3), B-cell lymphoma-2 (BCL2), BCLXL, BCL2-associated X (BAX), BCL2 associated agonist of cell death (BAD) and PRDX2 proteins in the cells of the two groups treated with ethanol for different durations as analyzed by immunoblotting. Quantitative data are presented as means±SEM for three independent experiments. Significantly different at *p<0.05, ***p<0.001.*

Mouse model of alcoholic liver injury. Male SV129 wild-type (WT) and *Prdx2* knockout (KO) mice, 20-25 g in weight, were obtained from the Korean Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea. They were bred and maintained in individually ventilated cages in the small animal feeding room of the Life Science and Technology College of Heilongjiang Bayi Agricultural Reclamation University at 22-25˚C under 40-60% relative humidity and 12-h day/12-h night conditions. All the animals were provided food and water *ad libitum*. In *Prdx2* KO mice, all six exons of *Prdx2* gene were replaced with the *neo* gene using traditional homologous recombination. The mice were randomly divided into six groups, with five mice in each group. The mice were gavaged with 31.5% alcohol at a dose of 5 g/kg body weight, three times in total, every 12 h.The animals in the control group were given the same dose of saline.

Statistical analysis. Data from *in vitro* experiments are presented as means±SEM of the means for triplicate samples. Statistical differences were analyzed by ANOVA or Student's *t*-test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Generation of stable PRDX2-silenced L02 hepatocyte cell line. GFP gene was introduced into the lentivirus vector as a marker gene. Using fluorescence microscopy, the cells transfected with lentivirus were observed, and those in mock and shPRDX2 groups emitting green GFP fluorescence were visualized; no such fluorescence was observed in the cells in the blank group (Figure 1A). The transfection rate, as determined by flow cytometry, was more than 95%, which indicated that lentivirus had successfully entered the cells (Figure 1B). The expression of *PRDX2* in the mock and sh*PRDX2* cells was determined using immunoblotting. The expression in the sh*PRDX2* group was significantly lower than that in the mock group (Figure 1C). To determine the effect of *PRDX2* knockdown on cell viability, growth curves were generated for the two groups of cells using MTT assay, and it was found that there was no significant difference in the cell viability between the groups. Thus, an L02 hepatocyte line with stable *PRDX2* gene silencing and the blank carrier cell line were successfully constructed *in vitro* (Figure 1D)

In order to investigate the toxicity of ethanol in the *PRDX2* knockdown cells, MTT assay of cells treated with different concentrations of ethanol was performed. Significant differences between mock and sh*PRDX2* groups were observed concentrations of ethanol of 400 mM and higher, suggesting that *PRDX2* knockdown can enhance the killing effect of ethanol. These results indicate that *PRDX2* is involved in alcohol-induced hepatocyte damage (Figure 1E).

Ethanol treatment affects the cell cycle of shPRDX2 L02 hepatocytes and induces G_1 phase arrest. The cells in the mock and sh*PRDX2* groups treated with 400 mM ethanol for 0, 6, and 12 h were used for cell-cycle analysis. The number of cells in the sub-G_0 phase was increased with increasing treatment duration, indicating an increase in apoptosis. Compared with the cells in mock group, those in the sh*PRDX2* group showed a significant increase in the sub-G_0 population. The decrease in the number of cells in the G_1 phase with the increase in the duration of treatment indicates that DNA replication was inhibited. Compared with mock group, in shPRDX2 group the number of cells in the G_1 phase was significantly reduced (Figure 2A). The percentages of cells in the sub-G_0 and G_1 phases of the cell cycle in the two groups are shown in Figure 2B. Immunoblot analysis revealed that the expression of cell-cycle inhibition protein, p21, was higher in the sh*PRDX2* group than in the mock group. In contrast, the expression levels of cyclin D1 and CDK4 decreased significantly in the sh*PRDX2* group compared to their levels in the mock group at the corresponding time points. Based on these results, it is believed that *PRDX2* gene silencing enhanced the inhibitory effect of ethanol on the progression of hepatocytes to the G_1

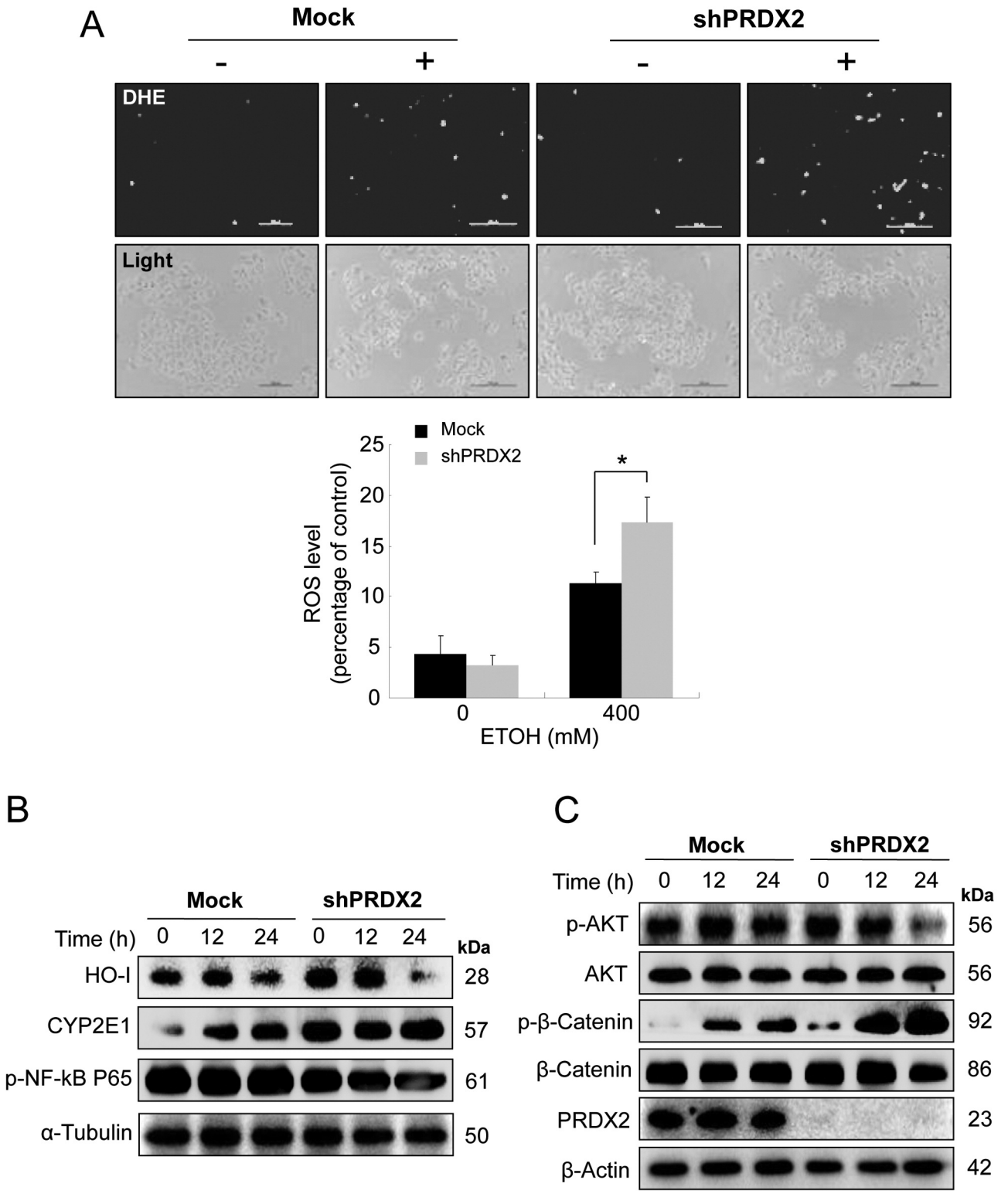


Figure 4. *Ethanol-induced apoptosis of peroxiredoxin II gene-silenced L02 (shPRDX2) L02 hepatocytes is related to reactive oxygen species (ROS) and protein kinase B (AKT) expression. A: The level of ROS l was observed under a fluorescence microscope after dihydroethidium (DHE) staining. The bar graph shows the ROS levels as means±SEM for three independent experiments. *Significantly different at p<0.05. B: The expression of heme oxygenase 1 (HO1), cytochrome P450 2E1 (CYP2E1), and phosphorylated nuclear factor kappa-B protein 65 (p-NF-κB P65) as analyzed by immunoblotting. C: The expression levels of phosphorylated protein kinase B (p-AKT), AKT, β-catenin, β-catenin, and PRDX2 as analyzed by immunoblotting.*

phase and increased the number of cells in the sub-G_0 phase (Figure 2C). The expression level of cell cycle-related proteins is shown in Figure 2D.

Ethanol activates mitochondria-dependent apoptosis in shPRDX2 L02 hepatocytes. The cell cycle is closely related to apoptosis. The increase in the number of cells in the sub-G_0 phase, and G_1 phase arrest indicates an increase in apoptosis. To confirm this, L02 cells in mock and sh*PRDX2* groups were treated with 400 mM ethanol for 0, 12, and 24 h and apoptosis was detected by fluorescence microscopy of annexin V-stained cells. Compared with the fluorescence intensity in mock group, that in the sh*PRDX2* group was significantly enhanced after ethanol treatment. Compared to the difference in the apoptosis of cells in the two groups after 12 h of ethanol treatment, the difference after 24 h of treatment was less, which might be due to prolonged treatment causing extensive apoptosis of cells in both the groups, thereby reducing the cell number (Figure 3A). Therefore, we further compared the effect of ethanol on the apoptosis of cells in mock and sh*PRDX2* groups at 0 and 12 h of treatment using flow cytometry. The apoptosis of sh*PRDX2* cells was increased significantly after the treatment (Figure 3B). To investigate the reason for the differences in apoptosis, the cells in mock and sh*PRDX2* groups were treated with 400 mM ethanol for 0, 12, and 24 h. Proteins extracted from these cells were assayed for the expression of apoptosis-related signal proteins. The levels of antiapoptotic proteins, BCL2 AND BCL-X_L, were reduced in the sh*PRDX2* group in a time-dependent manner and those of BAD, BAX, cleaved caspase-3, and cleaved PARP were increased. These results show that ethanol treatment further reduced the expression of mitochondrial anti-apoptotic proteins and increased the expression of pro-apoptotic proteins in L02 hepatocytes in the sh*PRDX2* group (Figure 3C).

Ethanol-induced apoptosis of shPRDX2 L02 hepatocytes is related to ROS and AKT. The cause for the difference in apoptosis was analyzed, and it was found that ROS were the main product of ethanol metabolism, whereas PRDX2 had the effect of scavenging ROS. Therefore, the difference in apoptosis might be related to ROS. The cells were treated with 400 mM ethanol for 12 h, and stained with dihydroethidium to detect the levels of ROS in the two groups of cells. It was found that the expression of ROS increased after ethanol treatment. Compared with the cells in mock group, the fluorescence intensity of the cells in the sh*PRDX2* group increased significantly after alcohol treatment (Figure 4A). Western blot analysis showed that the expression of CYP2E1, a key enzyme in alcohol metabolism, was increased. The expression of HO1 and NF-κB decreased. It is suggested that due to the absence of PRDX2 protein, ethanol enters the hepatocytes to produce a large amount of ROS, and the increase in ROS level causes oxidative damage (Figure 4B). To confirm this, the expression of the upstream proteins, AKT and β-catenin, was tested. It was observed that the phosphorylation of AKT decreased, whereas that of β-catenin increased significantly. It is suggested that ethanol may increase the level of ROS in sh*PRDX2* L02 cells through its metabolism, and mediate AKT phosphorylation to promote apoptosis (Figure 4C).

FGF2 inhibits the ethanol-induced apoptosis of shPRDX2 L02 hepatocyte line. To prove that AKT plays an important role in alcohol-induced apoptosis of *PRDX2*-silenced L02 hepatocyte line, the cells were pretreated with 20 ng/ml FGF2 (an AKT agonist) for 2 h. These cells were then treated with 400 mM ethanol for 12 h, and harvested. The apoptosis of mock and sh*PRDX2* cells was detected by flow cytometry (Figure 5A) and fluorescence microscopy (Figure 5B). Compared with the apoptosis of cells in the mock group, that in the sh*PRDX2* group was significantly increased and the fluorescence caused as a consequence of apoptosis was significantly enhanced. Under the same conditions, the expression levels of AKT/β-catenin and apoptosis-related proteins were determined. It was observed that pretreatment with FGF2 increased the phosphorylation level of AKT and reduced the phosphorylation level of β-catenin in mock and sh*PRDX2* groups. The increase in expression of apoptosis-related proteins induced by ethanol treatment was inhibited (Figure 5C). To further confirm the key role of PRDX2 in alcoholic liver injury, *Prdx2* knockout mice were used and a model of alcoholic liver injury was established by gavaging ethanol (5 g/kg body weight) three times at intervals of 12 h. The histopathological changes in the liver were detected by hematoxylin and eosin staining (Figure 5G). After ethanol treatment, the liver of PRDX2-deficient mice showed morphological changes; the membrane of hepatocytes was fuzzy, the nuclei were shrunken and the structure of hepatic cord around capillaries disappeared. Compared with wild-type mice, $Prdx2^{-/-}$ mice had more fat vacuoles and exhibited more hepatocyte damage (Figure 5D). The above results indicate that the inhibition of PRDX2 on alcohol-induced apoptosis of hepatocytes is mediated through the regulation of AKT/β-catenin signaling pathway.

Discussion

Alcoholic liver disease is caused by excessive intake of alcohol for a long time. Eighty percent of people who drink excessively suffer from fatty liver, and only 20% of them develop more serious liver diseases, such as hepatitis and liver fibrosis (15). Alcoholic liver disease mainly consists of three stages: Steatosis, alcoholic hepatitis, and chronic

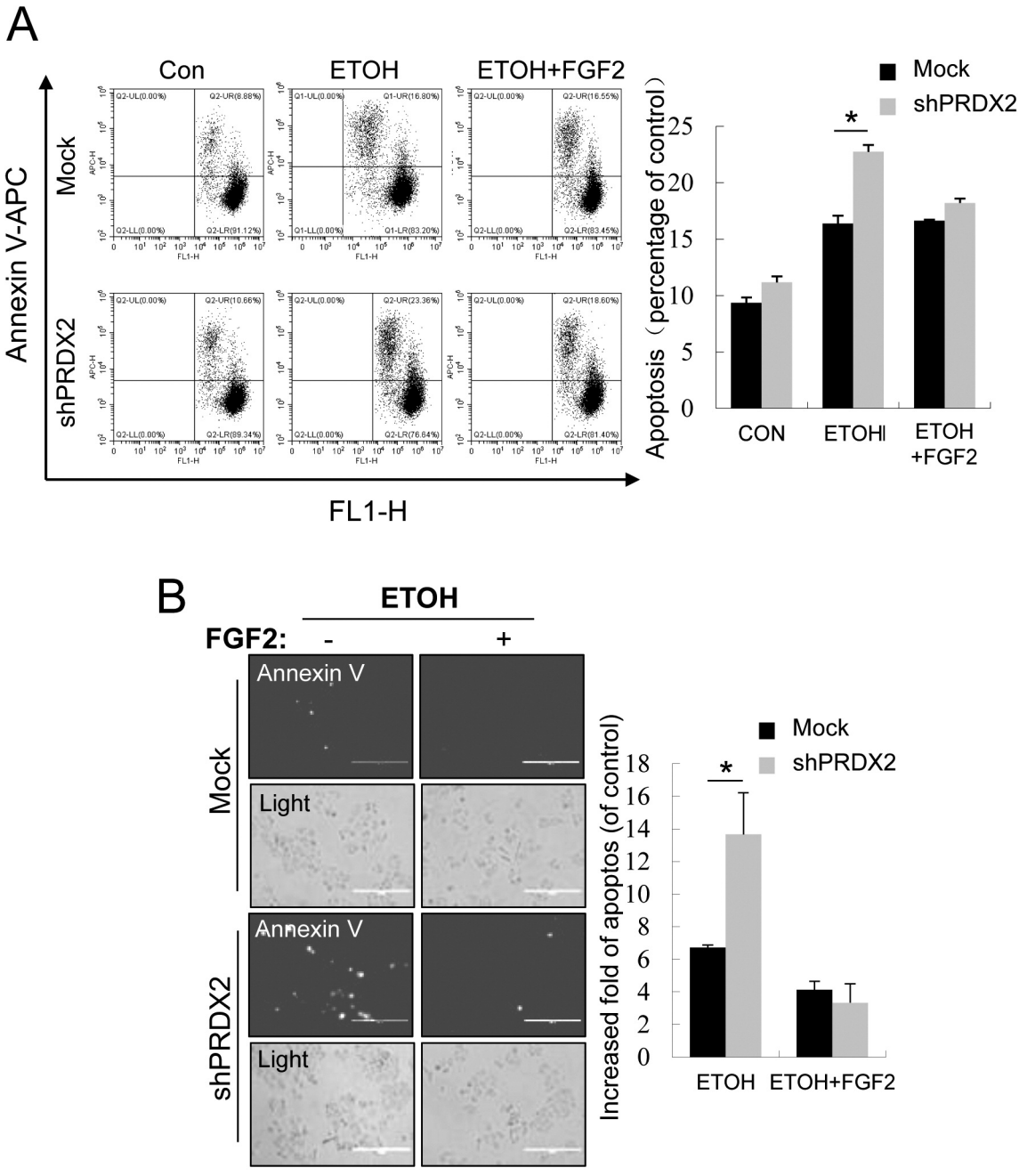


Figure 5. *Continued*

hepatitis with progressive fibrosis or cirrhosis (16). These stages may sometimes exist in an individual at the same time. At present, the exact pathophysiological mechanism of alcoholic liver disease is not clear. Oxidative stress and lipid peroxidation are considered to be the main mechanisms leading to alcoholic liver injury (8). ROS are the primary cause of oxidative stress in cells. They act as second messengers in cells and participate in a variety of activities. Upon excessive intake of ethanol, it is metabolized in the liver, and ROS is the main by-product of this metabolism (17). A number of studies have shown that the accumulation of ROS is the key to the progression of fatty liver to steatohepatitis and cirrhosis, which can cause degeneration of the fatty liver, infiltration of inflammatory cells, hepatomegaly, fibrosis, cirrhosis, and other pathological changes (18). PRDXs are enzymes

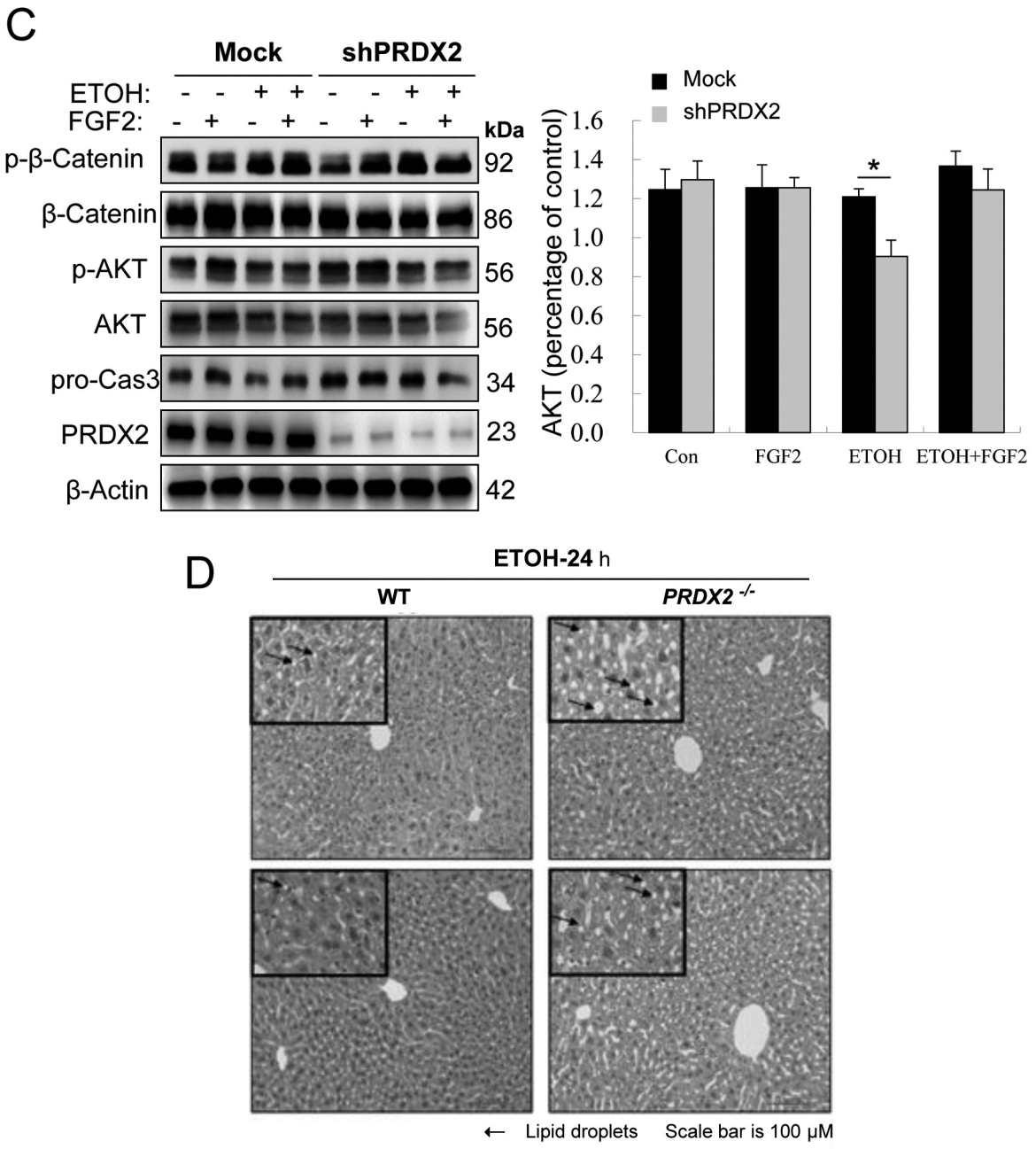


Figure 5. *Protein kinase B (AKT) agonist fibroblast growth factor 2 (FGF2) inhibits ethanol-induced apoptosis of peroxiredoxin II gene-silenced L02 (shPRDX2) L02 hepatocytes. A: Apoptosis was detected by flow cytometry and the percentage of apoptosis in mock and shPRDX2 groups was calculated. B: Apoptosis of cells in the two groups as determined by microscopy. C: The expression levels of pphosphorylated (p) β-catenin, β-catenin, p-AKT, AKT, pro-caspase 3 (pro-Cas3), and PRDX2 as assessed by immunoblotting. D: Histological assessment of liver injury in wild-type (WT) and Prdx2-null mice receiving alcohol treatment as shown by hematoxylin and eosin staining. Quantitative data are means±SEM for three independent experiments. *Significantly different at p<0.05.*

capable of scavenging ROS and have, therefore, become a hot topic of research on alcoholic liver in recent years. In 2009, it was shown that the expression of PRDX6 and ALDH2 was reduced in the liver of rats chronically fed alcohol, resulting in oxidative stress. It was also observed that PRDX2 protected the liver from oxidative damage induced by alcohol by coordinating with thioredoxin (19). Moreover, in kidney cells of mice showing early alcoholic

nephrotoxicity caused by chronic alcoholism, mitochondrial redox protein was altered, and PRDX3 and PRDX4 peroxidation led to renal oxidative stress (20). It was reported that fortilin, an anti-p53 molecule, prevented degradation of PRDX1 and inactivation of its enzymatic activity, and it was proposed that the interaction between fortilin and PRDX1 in the liver can be used to prevent alcohol-induced liver injury in clinics (21).

PRDX2 is a typical cysteine-containing enzyme, which mainly exists in the cytoplasm. The involvement of PRDX2 in alcoholic liver injury has not been reported. The results of the present study confirm that PRDX2 also plays an important role in alcohol-induced apoptosis of hepatocytes. When the hepatocytes were treated with alcohol, the apoptosis and the level of ROS increased significantly in both the groups, but the apoptosis and the level of active oxygen in the sh*PRDX2* group were significantly higher than those in mock group. The results showed that PRDX2 protected the hepatocytes from the injury caused by ethanol, presumably by clearing ROS produced by alcohol metabolism in the process of alcohol-induced apoptosis of hepatocytes.

It was found that the level of HO1 expression in sh*PRDX2* cells induced by ethanol was significantly lower than that in mock cells. The activity of HO1 has a direct effect on the antioxidant function of cells and reflects the degree of oxidative damage to cells. After 24 h of alcohol treatment, the expression of HO1 in *PRDX2* knockdown cells was significantly reduced, indicating that ROS produced by alcohol metabolism caused damage to liver cells after the loss of protection conferred by PRDX2, which is consistent with the results of the present study that ethanol treatment can inhibit the expression of antioxidant proteins in the liver (22).

CYP2E1 is an important enzyme in alcohol metabolism. It mainly exists in the endoplasmic reticulum and mitochondria of hepatocytes. It has high NADPH oxidase activity and can increase the production of ROS (23). The results showed that the expression level and activity of CYP2E1 protein in the liver and hepatocytes of mice increased after alcohol treatment. Based on the results of this study, PRDX2 may play a protective role in alcohol-induced apoptosis of hepatocytes. Therefore, further analysis of alcohol-treated PRDX2-silenced hepatocytes showed that the expression level of CYP2E1 protein in these cells was significantly higher than that in normal hepatocytes, which is consistent with this view.

Recent studies have shown that disorders in the PI3K/AKT signaling pathway can cause mitochondria-dependent apoptosis, and AKT/β-catenin signaling pathway is significantly inhibited in the process of alcohol-induced liver steatosis and apoptosis (24). Therefore, we speculate that AKT plays an important role in the alcohol-induced apoptosis of *PRDX2*-silenced cells. AKT is a serine/threonine kinase, which can be fully activated upon phosphorylation of its t308 and s473 sites. Activated AKT can regulate the phosphorylation of downstream substrates, including GSK3 and FOXOs, to regulate cell proliferation, differentiation, apoptosis, and other important processes. The results of the present study show that the phosphorylation level of AKT in the sh*PRDX2* group was significantly lower than that in the control group after alcohol treatment. Pretreatment of cells with the AKT agonist (FGF2) effectively improved the phosphorylation of AKT in cells. At the same time, with the addition of FGF2, the apoptosis induced by alcohol was significantly alleviated. This result shows that phosphorylation of AKT plays an important role in the apoptosis of hepatocytes, which can be regulated by PRDX2.

In the signal transduction pathway, AKT is an upstream signal protein, which can play a role by regulating the phosphorylation of downstream substrates, including glucogen synthase kinase 3 beta (GSK3β) and forkhead box O3 (25). β-Catenin is an important transcription regulator downstream of the GSK3β signaling pathway, and can also be regulated by AKT (26). The Wnt/β-catenin signaling pathway plays an important role in the proliferation of cells. When Wnt signal is not activated, β-catenin binds to the complex composed of adenomatous polyposis coli, axis inhibition protein and GSK3β phosphorylates β-catenin and is then ubiquitinated and degraded. When the Wnt signal is transmitted to the complex and its activity is inhibited. At this time, β-catenin cannot be phosphorylated by GSK3β, and is, therefore, free in the cytoplasm and accumulates in large amounts. It migrates to the nucleus and interacts with T cell-specific factor and other coactivating factors to mediate the expression of downstream target genes and promote the proliferation of cells (27). In alcohol-induced apoptosis, the phosphorylation of β-catenin was increased (24). This was also confirmed in the present study. Therefore, the results obtained in this study show that PRDX2 can regulate the apoptosis of hepatocytes through the AKT/β-catenin signaling pathway.

Overall, it can be concluded that PRDX2 plays a protective role in alcohol-induced apoptosis of hepatocytes, and this protective role is to regulate the phosphorylation of AKT signal protein, reduce the phosphorylation of β-catenin protein, and inhibit apoptosis by eliminating ROS produced by alcohol metabolism. These findings provide novel insights for research and treatment of alcoholic liver disease, and furnish a theoretical basis for further development and application of PRDX2 in alcoholic liver disease.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Y.H.H., W.L.L., M.H.J., H.N.S. and T.K. complete experiment and wrote the article. Y.H.J., Y.Q.Z., L.Z.K., L-Y.Y., J.W.H., H.N.S., T.K., and Y.H.H. performed data analysis. Y.H.H., J.K., H.N.S. and T.K had substantial contributions to conception and design. All Authors read and approved the final article.

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018R1A6A3A11051196, 2020R1I1A2052417) and grants from the Korean Research Institute of Bioscience and Biotechnology Research Initiative Program (KRIBB) (KGM5162021, RBM0112011).

Funding

This work was supported by the the "sanzong" project (ZRCPY201816) and scientific research team support plan of Heilongjiang Bayi Agricultural University (TDJH201904), China.

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Received June 3, 2020
Revised June 23, 2020
Accepted June 27, 2020