

Peroxiredoxin 1 has an anti-apoptotic role via apoptosis signal-regulating kinase 1 and p38 activation in mouse models with oral precancerous lesions

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Abstract. Peroxiredoxin 1 (Prx1) is important in the protection of cells from oxidative damage and the regulation of cell proliferation and apoptosis. Prx1 is overexpressed in oral precancerous lesions of oral leukoplakia (OLK) and oral cancer; however, the association between Prx1 expression and OLK pathogenesis remains unknown. The present study investigated the role of Prx1 and its molecular mechanisms in oxidative stress-induced apoptosis during the pathogenesis of OLK. Wild-type and Prx1 knockout mice were treated with 50 μ g/ml 4-nitroquinoline-1-oxide (4NQO) or 4NQO + H₂O₂ for 16 weeks to establish mouse models with tongue precancerous lesions. Apoptotic cells were detected using terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. The expression of Prx1, apoptosis signal-regulating kinase 1 (ASK1), phosphor-ASK1, p38 and phosphor-p38 was analyzed using immunohistochemical staining, and their mRNA expression levels were evaluated by reverse transcription quantitative polymerase chain reaction. The present results demonstrated that 4NQO or 4NQO + H₂O₂ induced the development of tongue precancerous lesions in Prx1 knockout and wild-type mice. Prx1 was overexpressed in tongue precancerous lesions compared with normal tongue mucosa. There was a significant decrease in the degree of moderate or severe epithelial dysplasia, and mild epithelial dysplasia was clearly elevated, in Prx1 knockout mice treated with 4NQO + H₂O₂ compared with wild-type mice treated with 4NQO + H₂O₂. Prx1 suppressed apoptosis and upregulated phosphor-ASK1 and phosphor-p38 expression in tongue precancerous lesions. The present results suggest that Prx1 suppresses oxidative

stress-induced apoptosis via the ASK1/p38 signalling pathway in mouse tongue precancerous lesions. In conclusion, Prx1 and H₂O₂ have a coordination role in promoting the progression of tongue precancerous mucosa lesions. The present findings provide novel insight into Prx1 function and the mechanisms of Prx1 in OLK pathogenesis.

Introduction

Oral leukoplakia (OLK) is the most common oral precancerous lesion, with a global prevalence of 1% (1) and a malignant transformation rate of 0.13-17% (2). Following OLK transformation to oral cancer, the 5- and 10-year survival rates are 59 and 48%, respectively (3). Currently, the pathogenesis of OLK is unclear. Numerous studies have demonstrated that OLK is closely associated with smoking, drinking and betel chewing (4-8). Tobacco, betel nut and alcohol all increase the expression of the oxidant H₂O₂ in saliva and oral mucosal cells (9,10), and H₂O₂ expression at a high level may result in oxidative damage of DNA and activation of apoptotic genes, thus inducing apoptosis of cells (11-13). Reactive oxygen species (ROS) is a collective term that describes O₂-derived non-radical species, including H₂O₂, and O₂-derived free radicals, such as superoxide anion, hydroxyl and peroxy free radicals. At physiological low levels, ROS functions as redox messengers in intracellular signaling and regulation. However, excessive ROS induce oxidative modification of macromolecules, inhibit protein functions and promote apoptosis of cells (14).

Peroxiredoxins (Prxs) are thio-specific antioxidant enzymes, and may be induced by several types of oxidative stress conditions. They are associated with neutralizing cellular hydroperoxides, which protect cells from oxidative damage. Prxs are often identified in mammals, yeast and bacteria, which are classified as 1-cys Prx and 2-cys Prx on the basis of one or two conserved cysteine residues. Peroxiredoxin 1 (Prx1), as an important member of Prxs, has two conserved cysteine residues (15). Current evidence suggests that Prx1, as a simple peroxidase, initiates the mechanistic switch from peroxidase to chaperone function, meaning that it is closely associated with a variety of biological processes including cell proliferation, differentiation and apoptosis (16).

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Yanagawa *et al* (17,18) have identified that an overexpression of Prx1 is significantly associated with the recurrence of oral squamous cell carcinoma (OSCC). Previous studies by the present authors have confirmed that Prx1 expression and 8-hydroxy-2'-deoxyguanosine (8-OHdG) expression levels are elevated in human OLK tissues, and an increase in 8-OHdG is consistent with the expression of Prx1 (19). This result indicates that there is a significant association between Prx1 and oxidative damage in the progression of OLK. Whether Prx1 is important in OLK remains unknown, and the mechanism associated with Prx1 and apoptosis or oxidative stress remains unclear.

Apoptosis signal-regulating kinase 1 (ASK1) is a serine-threonine protein kinase that functions as a mitogen-activated protein kinase (MAPK), which activates c-Jun N-terminal kinase (JNK) and p38 MAPK signaling cascades. ASK1 may be activated by various stresses and is critical in the regulation of signaling in response to oxidative stress, which is a major contributor to cell death (20-22). Kim *et al* (23) have demonstrated that Prx1 plays a negative role in regulating ASK1-induced apoptosis. However, to the best of our knowledge, there is no evidence that reveals similar results *in vivo*.

In the present study, 4-nitroquinoline-1-oxide (4NQO) was used to establish a precancerous lesion model in wild-type and Prx1 knockout mice, to investigate the apoptotic role of Prx1 in oral precancerous lesions based on the hypothesis that Prx1 may mediate the ASK1/p38 signalling pathway. In addition, the effect of oxidative stress on Prx1 and apoptosis in oral precancerous lesions was also determined. Understanding the molecular mechanisms of Prx1 involved in the initiation and progression to malignancy may benefit methods for the prognosis and treatment of oral precancerous lesions.

Materials and methods

Experimental animals. A total of 50 wild-type C57BL/6 mice (Vital River Laboratory Animal Technology Co., Ltd., Shenzhen, China) and 50 Prx1 knockout mice, which had been previously established (24), aged 6-8 weeks old, were used in the present study. All the animals were kept in accordance with institutional guidelines in specific pathogen free units at 24±2°C room temperature with 40-60% humidity, in a 14 day light/10 day dark cycle with freely accessible water food. The experimental protocol for the present study was approved by the local Ethical Committee for Animal Use. The experimental mice were randomly divided into six groups that underwent various treatments as follows: Wild-type control (n=10), treatment with vehicle (distilled water); wild-type 4NQO group (n=20), treatment with 50 µg/ml 4NQO (Sigma-Aldrich, St. Louis, MO, USA) every day; wild-type 4NQO + H₂O₂ group (n=20), treatment with 50 µg/ml 4NQO every day and 3% H₂O₂ smeared on tongue mucosa three times a week; Prx1 knockout control group (n=10), treatment with vehicle (distilled water); Prx1 knockout 4NQO group (n=20), treatment with 50 µg/ml 4NQO every day; and Prx1 knockout 4NQO + H₂O₂ group (n=20), treatment with 50 µg/ml 4NQO every day and 3% H₂O₂ smeared on tongue mucosa three times a week. All these treatments lasted for 16 weeks. The mice were euthanized and the tongues were resected and immediately stored in liquid

nitrogen for future molecular/cellular analysis, or in formalin for the preparation of paraffin-embedded tissue blocks.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Apoptosis was examined using *In Situ* Cell Death Detection kit, POD (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol. The paraffin-embedded tissues were baked at 65°C for 1 h, de-waxed using xylene and gradually dehydrated with 100, 95, 90, 80 and 70% ethanol. The specimens were washed twice with phosphate-buffered saline (PBS) for 5 min each wash, treated with proteinase K solution (10 mM Tris-HCl with 20 µg/ml proteinase K; Merck Millipore, Darmstadt, Germany), incubated at 37°C for 15 min, and washed twice with PBS for 5 min each wash. Dry specimens were treated with 50 µl TUNEL reaction mixture (dilution, 1:5), covered with a cover slip, hydrated in light-free conditions and incubated at 37°C for 60 min. The specimens were subsequently washed three times with PBS for 5 min each wash, and dry specimens were treated with 50 µl converter-POD, covered with a cover slip, hydrated in light-free conditions, incubated at 37°C for 60 min, and washed three times in PBS for 5 min each wash. Finally, the specimens were subjected to incubation with freshly prepared 3,3'-diaminobenzidine (DAB) solution for 10 min, hematoxylin staining, soaking twice in anhydrous ethanol for 5 min and xylene for 2 min and mounting with neutral gum.

Immunohistochemical staining. The paraffin-embedded mouse tongue specimens (4 µm) were de-paraffinized and hydrated using gradient alcohol, and rinsed with PBS. Antigen retrieval for Prx1, ASK1, phosphor-ASK1 and p38 was conducted with a citrate buffer (pH=6.0) in a microwave oven, and for phosphor-p38 with an EDTA buffer. Subsequently, the sections were blocked with 3% H₂O₂ at room temperature for 15 min to remove the endogenous peroxidase and incubated in 10% goat serum (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) as a blocking solution at 37°C for 30 min. The specimens were incubated with the following primary antibodies: Polyclonal rabbit anti-Prx1 (dilution, 1:5,000; #ab41906; Abcam, Cambridge, MA, USA), polyclonal rabbit anti-ASK1 (dilution, 1:200; #bs-1425R; Bioss, Inc., Beijing, China), monoclonal rabbit anti-phosphor-ASK1 (dilution, 1:400; #GTX50229; GeneTex, Inc., Irvine, CA, USA), p38 (dilution, 1:800; #bs-0637R; Bioss, Inc.) and phosphor-p38 (dilution, 1:200; #4631; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4°C overnight. The specimens were incubated with biotinylated secondary IgG antibody (from the MaxVision™ HRP-Polymer anti-Mouse IHC kit; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) at 37°C for 30 min, and then visualized using DAB staining for 2-5 min. The specimens were subjected to Mayer's hematoxylin staining, dehydration and mounting. For the negative control, PBS was used in place of a primary antibody. Hepatocellular carcinoma tissue and small intestine tissue were used as the positive controls for Prx1 and p38, respectively, while breast carcinoma tissue was used as the positive control for ASK1, phosphor-ASK1 and phosphor-p38.

For evaluating the apoptosis level and the expression of phosphor-p38, the cells with positive staining were

determined by counting the stained cells using Image-Pro Plus version 7.0 (Media Cybernetics, Inc., Rockville, MD, USA). In total, ~1,000 cells were counted for each tumor specimen. In order to evaluate the expression of Prx1, ASK1, p38 and phosphor-ASK1, the stained cells from three to five representative microscope fields were counted for each specimen (magnification, x200) and the mean optical density (MOD) was calculated for each mouse tongue tissue using Image-Pro Plus version 7.0 software as follows: MOD = integrated option density / area.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from mouse tongue tissues using TRIzol Reagent (Invitrogen™; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA was synthesized by reverse transcribing 2 µg RNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems®; Thermo Fisher Scientific, Inc.). In total, 1 µl aliquots of cDNA were used as the templates for qPCR. Sequences for all target gene primers were synthesized by Sangon Biotech (Shanghai, China) as follows: Prx1, forward: 5'-AATGCAAAAATTGGGTATCCTGC-3' and reverse 5'-CGTGGGACACACAAAAGTAAAGT-3'; ASK1, forward: 5'-AAGTCCCAACCCATAGAAATTCCT-3' and reverse 5'-AGCCAGTCGGTAAGTTCAGAATCTT-3'; p38, forward 5'-GAGCTGAAGATTCTGGATTTTGG-3' and reverse 5'-TAGCCACGTAGCCGGTCATT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-AGGTCG GTGTGAACGGATTG-3' and reverse 5'-TGTAGACCA TGAGTTGAGGTCA-3'. The cycling conditions for RT-PCR were as follows: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. The UltraSYBR Mixture (With ROX) (ComWin Biotech Co., Ltd., Beijing, China) was used for qPCR, and the cycling conditions were as follows: 95°C for 10 min, 95°C for 15 sec and 60°C for 15 sec for 40 cycles. For data analysis, the 2^{-ΔΔC_q} method (25) was used for the normalization of the genes of interest against GAPDH. The experiments were conducted three times.

Statistical analysis. Statistically significant differences were analyzed by χ^2 , two-tailed Student's t-test and Kruskal-Wallis one-way analysis of variance test. Bonferroni was used as a post-hoc test. SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for analysis. $P < 0.05$ was considered to indicate a statistically significant difference. $P < 0.017$ was considered to indicate a statistically significant difference in the Bonferroni test.

Results

Tongue precancerous lesion model established in Prx1 knockout mice. 4NQO was used to induce the development of tongue precancerous lesions in Prx1 knockout and wild-type mice. No tongue precancerous lesions were observed in the control mice at the end of the 16th week, while in Prx1 knockout and wild-type mice treated with 4NQO or 4NQO + H₂O₂ the tongues of the mice exhibited white, thick, rough and visible white patches as well as surface toughness. Histological observation revealed epithelial dysplasia with varying degrees and OSCC on the tongues, indicating that the model of tongue

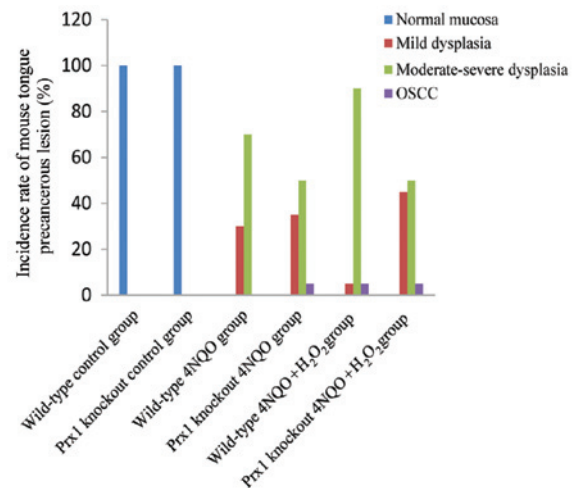


Figure 1. Incidence and type of mouse tongue precancerous lesions in six experimental mouse models used in the present study. Control groups, n=10; other groups, n=20. Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide; OSCC, oral squamous cell carcinoma.

precancerous lesions in Prx1 knockout mice was successfully established. There was a significant decrease in the degree of moderate or severe epithelial dysplasia ($P=0.016$), and mild epithelial dysplasia was clearly elevated ($P=0.011$), in Prx1 knockout mice treated with 4NQO + H₂O₂ compared with wild-type mice treated with 4NQO + H₂O₂ (Fig. 1; Table I). The application of 3% H₂O₂ alone (3 times/week) did not induce epithelial dysplasia of tongue mucosa over 16 weeks (data not shown). These results indicated that Prx1 and H₂O₂ play a coordination role in promoting the progression of tongue precancerous lesions.

Prx1 is over-expressed in tongue precancerous lesions. The expression of Prx1 was analyzed by RT-qPCR and immuno-histochemical staining. The mRNA expression of Prx1 was increased in the wild-type 4NQO group compared with the wild-type control group ($P=0.046$). The mRNA expression level of Prx1 was also increased in the wild-type 4NQO + H₂O₂ group compared with the wild-type control group ($P=0.009$). There was no statistically significant difference in mRNA expression between the wild-type 4NQO and 4NQO + H₂O₂ groups (Fig. 2A). The protein expression levels of Prx1 were increased in the 4NQO and 4NQO + H₂O₂ groups compared with mice from the wild-type control group ($P=0.035$ and $P=0.024$, respectively). The expression of Prx1 in the 4NQO + H₂O₂ group was increased compared with the 4NQO group, but this was not statistically significant ($P=0.847$; Fig. 2B). These results indicate that Prx1 may be important in promoting cell proliferation in oral precancerous lesions.

Prx1 knockout increases cell apoptosis in tongue precancerous lesions. The apoptotic rate in the wild-type 4NQO group was elevated compared with the wild-type control group ($P < 0.001$). The apoptotic rate in the wild-type 4NQO + H₂O₂ group was decreased compared with the 4NQO group ($P=0.004$). An increased apoptotic rate in the Prx1 knockout 4NQO and Prx1 knockout 4NQO + H₂O₂ groups was observed compared with the wild-type 4NQO ($P=0.009$) and wild-type

Table I. Incidence and type of mouse tongue precancerous lesions in six experimental mouse models.

Group	n	Normal mucosal, n (%)	Mild dysplasia, n (%)	Moderate-severe dysplasia, n (%)	OSCC, n (%)
Total	100				
Wild-type control	10	10 (100)	0 (0)	0 (0)	0 (0)
Prx1 knockout control	10	10 (100)	0 (0)	0 (0)	0 (0)
Wild-type 4NQO	20	0 (0)	6 (30)	14 (70)	0 (0)
Prx1 knockout 4NQO	20	0 (0)	7 (35)	12 (50)	1 (5)
Wild-type 4NQO + H ₂ O ₂	20	0 (0)	1 (5)	18 (90)	1 (5)
Prx1 knockout 4NQO + H ₂ O ₂	20	0 (0)	9 (45) ^a	10 (50) ^a	1 (5)

^aP<0.05 vs. wild-type 4NQO + H₂O₂ group. OSCC, oral squamous cell carcinoma; Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide.

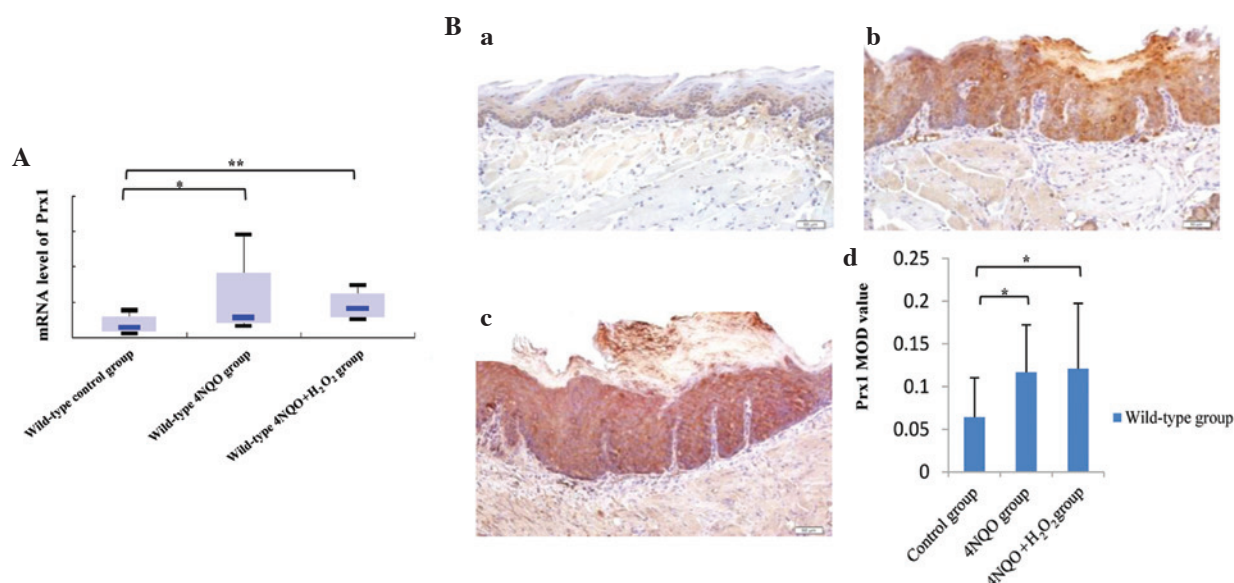


Figure 2. Prx1 is over-expressed in tongue precancerous lesions. (A) mRNA level of Prx1 in wild-type mice determined by reverse transcription polymerase chain reaction. (B) Positive expression of Prx1 in mouse tongue premalignant lesions in the (a) wild-type control group, (b) wild-type 4NQO group and (c) wild-type 4NQO + H₂O₂ group (magnification, x200). (d) Prx1 MOD value. *0.01<P<0.05; **0.001<P<0.01. Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide; MOD, mean optical density.

4NQO + H₂O₂ groups (P=0.024), respectively. These results indicate that Prx1 inhibits apoptosis in tongue precancerous lesions (Fig. 3A and B).

Prx1 knockout results in the downregulation of ASK1. In order to evaluate the effect of Prx1 on the activation of ASK1 in tongue precancerous lesions, the expression of total ASK1 and phosphor-ASK1 was observed in Prx1 knockout and wild-type mice. The present results demonstrated that the mRNA expression level of ASK1 was increased in wild-type 4NQO and wild-type 4NQO + H₂O₂ groups compared with the wild-type control group (P=0.001 and P=0.002, respectively; Fig. 4A). A statistically significant difference in the mRNA expression level of ASK1 between wild-type 4NQO and 4NQO + H₂O₂ groups was observed. The mRNA expression level of ASK1 was increased in Prx1 knockout control group compared with wild-type control group (P=0.003; Fig. 4Ba). The mRNA expression level of ASK1 in Prx1 knockout 4NQO and Prx1 knockout 4NQO + H₂O₂ groups

was increased compared with the wild-type group, although this was not statistically significant (P=0.704 and P=0.24, respectively; Fig. 4Bb and c).

Immunohistochemical analysis revealed that there was no statistically significant difference in protein expression of ASK1 between any groups (Fig. 4C). Compared with the wild-type 4NQO group, the expression of phosphor-ASK1 was decreased in the Prx1 knockout 4NQO group (P=0.022). A similar expression pattern was observed in wild-type and Prx1 knockout 4NQO + H₂O₂ groups (P=0.001). There was no significant difference in the phosphorylation of ASK1 in the wild-type 4NQO and wild-type 4NQO + H₂O₂ groups compared with the wild-type control group (P=0.481 and P=0.104), suggesting that phosphor-ASK1 has a positive association with Prx1 expression (Fig. 4D).

Prx1 knockout suppresses the expression of p38. In order to evaluate the effect of Prx1 on the activation of p38 MAPK in tongue precancerous lesions, the expression of total p38 and

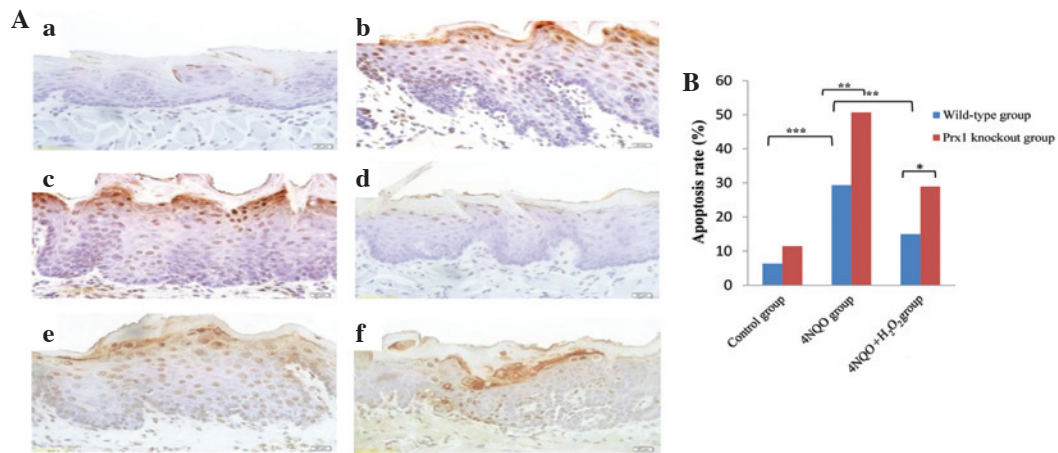


Figure 3. Prx1 knockout increases cell apoptosis in tongue precancerous lesions. (A) Terminal deoxynucleotidyl transferase dUTP nick-end labeling assay in (a) wild-type control group, (b) wild-type 4NQO group, (c) wild-type 4NQO + H₂O₂ group, (d) Prx1 knockout control group, (e) Prx1 knockout 4NQO group and (f) Prx1 knockout 4NQO + H₂O₂ group (magnification, x400). (B) Prx1 negatively regulated the apoptotic rate of cells in mouse tongue premalignant lesions. *0.01<P<0.05; **0.000<P<0.01; ***P=0.000. Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide.

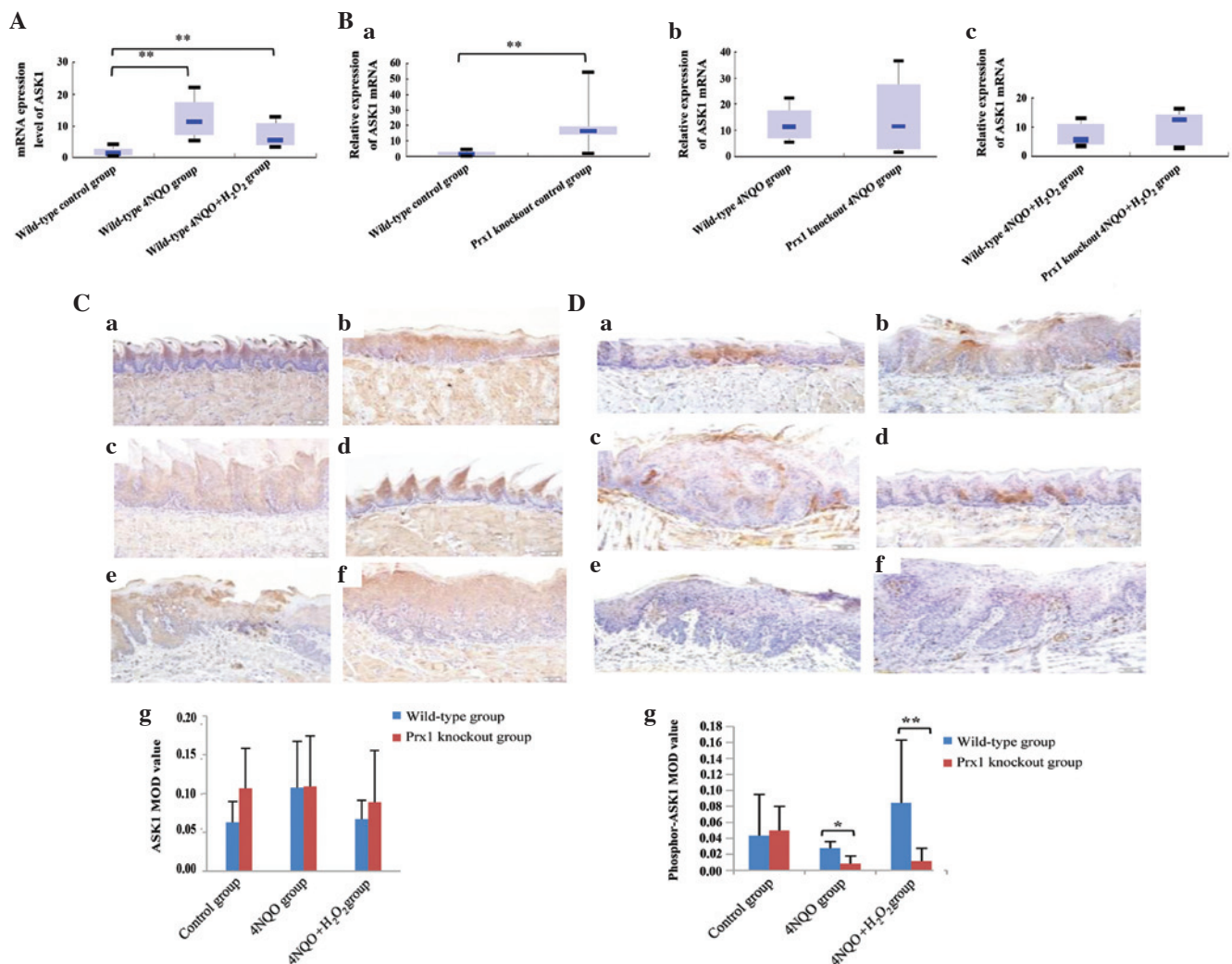


Figure 4. Prx1 knockout leads to a downregulation of ASK1. (A) mRNA expression level of ASK1 was elevated in mouse tongue premalignant lesions, as determined by RT-qPCR. (B) RT-qPCR determination of the relative expression of ASK1 mRNA in Prx1 knockout mice (a) control group, (b) 4NQO group and (c) 4NQO + H₂O₂ group. (C) Prx1 had no clear association with ASK1 in mouse tongue premalignant lesions in the (a) wild-type control group, (b) wild-type 4NQO group, (c) wild-type 4NQO + H₂O₂ group, (d) Prx1 knockout control group, (e) Prx1 knockout 4NQO group and (f) Prx1 knockout 4NQO + H₂O₂ group (magnification, x200). (g) ASK1 MOD value. (D) Prx1 positively regulated the activation of phosphor-ASK1 in mouse tongue premalignant lesions in the (a) wild-type control group, (b) wild-type 4NQO group, (c) wild-type 4NQO + H₂O₂ group, (d) Prx1 knockout control group, (e) Prx1 knockout 4NQO group and (f) Prx1 knockout 4NQO + H₂O₂ group (magnification, x200). (g) Phosphor-ASK1 MOD value. *0.01<P<0.05; **0.000<P<0.01. Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide; ASK-1, apoptosis signal-regulating kinase 1; MOD, mean optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

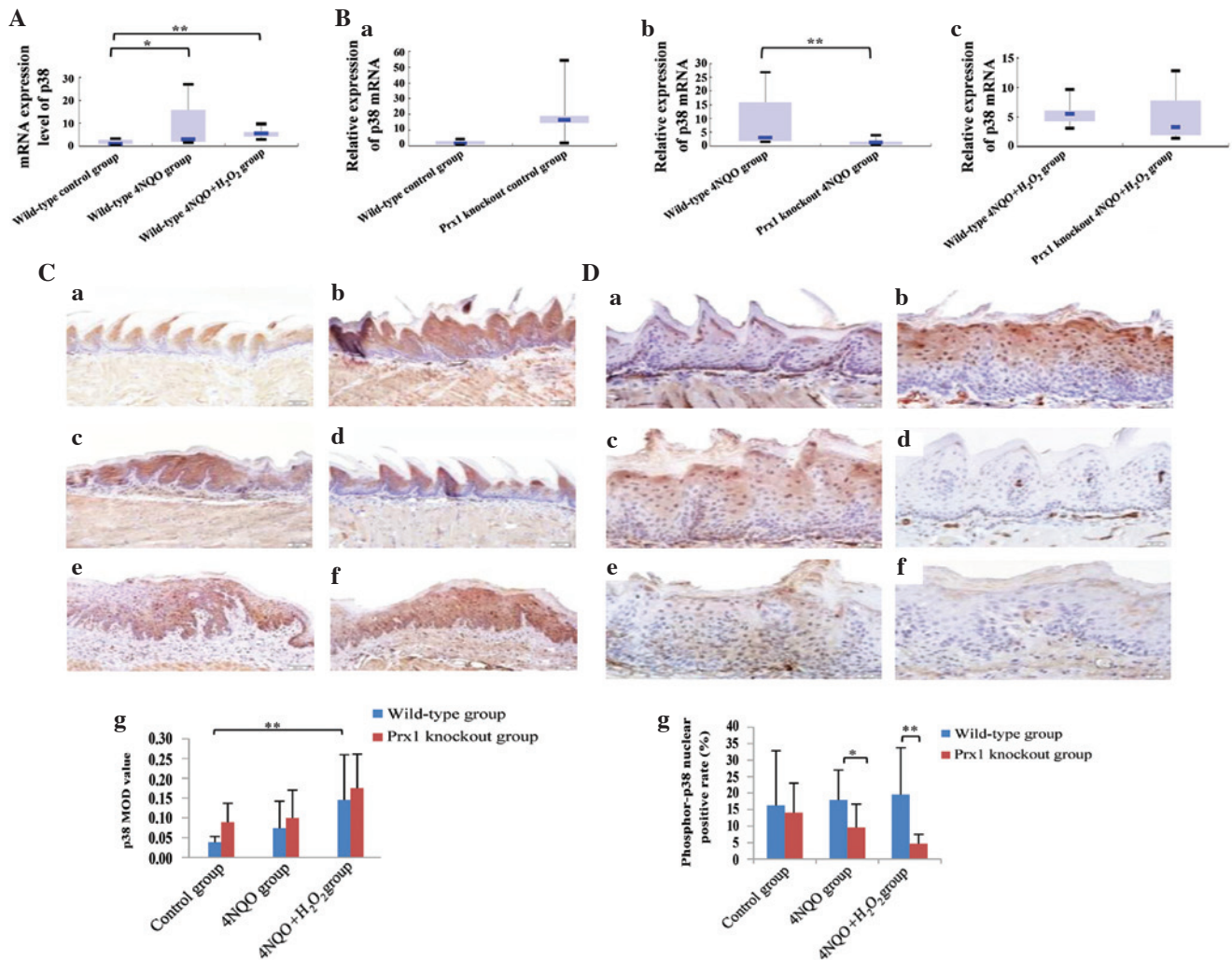


Figure 5. Prx1 knockout suppresses the expression of p38. (A) mRNA expression level of p38 was elevated in mouse tongue premalignant lesions, as determined by RT-qPCR. (B) RT-qPCR determined the relative expression level of p38 mRNA in Prx1 knockout mice (a) control group, (b) 4NQO group and (c) 4NQO + H₂O₂ group. (C) Prx1 positively regulated the activation of p38 in mouse tongue premalignant lesions in the (a) wild-type control group, (b) wild-type 4NQO group, (c) wild-type 4NQO + H₂O₂ group, (d) Prx1 knockout control group, (e) Prx1 knockout 4NQO group and (f) Prx1 knockout 4NQO + H₂O₂ group (magnification, x200). (g) p38 MOD value. (D) Prx1 positively regulated the activation of phosphor-p38 in mouse tongue premalignant lesions in the (a) wild-type control group, (b) wild-type 4NQO group, (c) wild-type 4NQO + H₂O₂ group, (d) Prx1 knockout control group, (e) Prx1 knockout 4NQO group and (f) Prx1 knockout 4NQO + H₂O₂ group (magnification, x400). (g) Nuclear positive rate of phosphor-p38. *0.01<P<0.05; **0.000<P<0.01. Prx1, peroxiredoxin 1; 4NQO, 4-nitroquinoline-1-oxide; MOD, mean optical density; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

phosphor-p38 was detected in Prx1 knockout and wild-type mice. The mRNA expression level of p38 was increased in the wild-type 4NQO and 4NQO + H₂O₂ groups compared with the wild-type control group ($P=0.021$ and $P=0.001$, respectively). The difference in mRNA expression levels of p38 between wild-type 4NQO and 4NQO + H₂O₂ groups was not statistically significant ($P=0.401$; Fig. 5A). The mRNA expression level of p38 was decreased in the Prx1 knockout 4NQO group compared with the wild-type 4NQO group ($P=0.006$). The mRNA expression of p38 was decreased in the Prx1 knockout 4NQO + H₂O₂ group, although no statistically significant difference was observed with the wild-type 4NQO + H₂O₂ group ($P=0.649$; Fig. 5B).

The protein expression of p38 was clearly increased in the wild-type 4NQO + H₂O₂ group compared with the wild-type control group ($P=0.002$; Fig. 5C). The level of phosphor-p38 was decreased in the Prx1 knockout 4NQO group compared with the wild-type 4NQO group ($P=0.022$). The same expression pattern

was observed in Prx1 knockout and wild-type 4NQO + H₂O₂ groups ($P=0.001$). The expression level of phosphor-p38 was not significantly different in the wild-type 4NQO and 4NQO + H₂O₂ groups compared with the wild-type control groups ($P=0.606$ and $P=0.333$, respectively), indicating that phosphor-p38 had a clear positive association with the expression of Prx1 (Fig. 5D).

Discussion

OLK is the most common oral precancerous lesion, which may undergo a carcinomatous change to OSCC (26). At present, little is known concerning the pathogenesis of OLK. Previous studies have demonstrated that cell apoptosis is suppressed by Prx1 via the ASK1-mediated signaling pathway in human embryonic kidney 293 and cervical cancer HeLa cells (23). In a previous study by the present authors, an increased level of apoptosis was observed in OLK tissues, and Prx1 knockdown significantly enhanced the level of apoptosis in dysplastic oral keratinocyte

cells (data not shown). However, even though there are numerous *in vitro* studies concerning Prx1 and cell apoptosis, there are few *in vivo* studies. The present study has for the first time, to the best of our knowledge, designed carcinogenic experiments *in vivo* to observe the effect of Prx1 on cell apoptosis during the initiation and progression to malignancy of oral mucosa. In order to confirm the role of Prx1 in oral precancerous lesions *in vivo*, the present study established tongue precancerous lesion mouse models in Prx1 knockout mice and investigated whether Prx1 suppresses apoptosis induced by oxidative stress. The present study elucidated the possible molecular mechanism during the pathogenesis and development of oral precancerous lesions.

In eukaryotic cells, four MAPK signal transduction pathways, including extracellular signal-regulated kinase (ERK) 1/2, JNK, p38 and ERK5, have been identified. ERK1/2, JNK and p38 pathways are typical MAPK signal transduction pathways. Furthermore, JNK and p38 signaling pathways are associated with cell apoptosis (27-29). ASK1 is known as a proapoptotic, stress-activated signaling molecule, and is an ubiquitously expressed serine-threonine protein kinase that functions as a MAPK kinase to activate JNK and p38 MAPK signaling cascades (30). Prx1 is the most abundant and ubiquitously distributed member of the mammalian Prx family. It has been implicated in regulating cell proliferation, differentiation and apoptosis (31,32). ASK1 interacts with Prx1 in the presence of H₂O₂-induced stress and is negatively regulated by Prx1 (23). Nakagawa *et al* (33) have demonstrated that the activation of JNK and p38 is attenuated and hepatocarcinogenesis is increased in ASK1-deficient mice. Yan *et al* (34) have identified that ASK1 activated by arsenic trioxide in leukemic cells may play an antiapoptotic role, and Park *et al* (35) have demonstrated that *Bacillus anthracis* induces the apoptosis of activated macrophages by inhibiting the p38 MAPK pathway.

In the present study, the apoptotic rate of cells increased and the expression of phosphor-ASK1 and phosphor-p38 was downregulated in tongue precancerous lesions of Prx1 knockout mice. These results demonstrate that apoptosis suppression by Prx1 may be associated with the phosphorylation of ASK1 and p38, and that Prx1 has a positive regulatory role in the phosphorylation of ASK1 and p38. In addition, the present study also detected the transcription level of Prx1, ASK1 and p38 compared with that in normal epithelium, and the expression of Prx1, ASK1 and p38 was clearly increased in precancerous lesions compared with normal epithelium. When Prx1 was knocked-down, the ASK1 transcription level was significantly increased in the control group, indicating that Prx1 clearly inhibits the transcription of ASK1 in normal mucosa. By contrast, a knockdown of Prx1 resulted in a significant downregulation of p38 at a transcriptional level in the precancerous lesions, suggesting that Prx1 also positively regulates p38 in precancerous lesions. Overall, Prx1 suppresses oxidative stress-induced apoptosis in tongue precancerous lesions by positively regulating ASK1 and p38 expression at a molecular level.

In the present study, in the Prx1 knockout 4NQO + H₂O₂ mice, the degree of moderate to severe epithelial dysplasia was significantly reduced and mild epithelial dysplasia was clearly elevated compared with wild-type 4NQO + H₂O₂ mice. This suggests that Prx1 enhances cell proliferation during the pathogenesis of oral precancerous lesions. Therefore,

when oral precancerous lesions are affected by oxidative stress, Prx1 is important in inhibiting oxidative damage and apoptosis of cells, and promotes the progression of tongue precancerous lesions. Lee *et al* (36) have also demonstrated that Prx1 knockout results in the decrease of cell proliferation, and Prx1 is associated with tumor size, micro-vessel invasion and Edmonson tumor grade (37). In addition, microRNA-510 directly binds to the 3'-untranslated region of Prx1 and blocks its protein expression, leading to a suppression in the migration of human breast cancer cells (38).

In the present study, the application of H₂O₂ alone as an oxidative stressor had no obvious effect on lesion development. However, more severe lesions were observed in mice from the wild-type 4NQO + H₂O₂ group compared with mice from the wild-type 4NQO group, indicating that H₂O₂ application coupled with 4NQO has a positive effect on promoting the development and progression of lesions. H₂O₂ is known as the most common member of ROS and induces apoptosis in various types of malignancies (39,40). However, in the present study, compared with 4NQO-induced tongue precancerous lesions, cell apoptosis was moderately reduced in mice from the 4NQO + H₂O₂ group. A similar pattern was observed in Prx1 knockout mice. Previous studies have revealed that 4NQO treatment leads to the formation of H₂O₂ superoxide and hydroxyl radicals, thus resulting in the production of a substantial amount of 8-OHdG in DNA and oxidative damage in normal human fibroblasts (41). Tang *et al* (42) have demonstrated that H₂O₂ preconditioning at low concentrations may protect rat pheochromocytoma PC12 cells from apoptosis induced by H₂O₂. In addition, oxidant preconditioning protects human proximal tubular cells against lethal oxidant injury (43). In the present study, 3% H₂O₂ was applied to mouse tongue mucosa three times a week during the development of 4NQO-induced mouse tongue precancerous lesions for 16 weeks. The 3% H₂O₂ was revealed to be a mild stimulus compared with 50 µg/ml 4NQO. The 3% H₂O₂ treatment may alleviate apoptosis induced by subsequent 4NQO exposure in tongue mucosa epithelia of the mice. These data indicate that H₂O₂ at a low concentration may inhibit apoptosis. A previous study also revealed that H₂O₂ at a low concentration promotes cell proliferation (44). A low dose of H₂O₂ was able to reverse DHM-induced cell apoptosis of human hepatocellular carcinoma (44). This may indicate that the balance between ROS production and various antioxidants is vitally important for cancer cell growth.

The present carcinogenic *in vivo* experiments were used to observe the effect of Prx1 on cell apoptosis during the development and progression to malignancy of mouse tongue mucosa. The present study concludes that Prx1 may suppress oxidative stress-induced apoptosis via the ASK1/p38 signaling pathway in mouse tongue precancerous lesions, and H₂O₂ and 4NQO play a coordination role in promoting the progression of tongue mucosa precancerous lesions. In addition, H₂O₂ at a low concentration level may inhibit apoptosis. The present findings provide novel insights into Prx1 function and the mechanisms of OLK pathogenesis.

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