



OPEN Protective effects of isorhamnetin against H₂O₂-induced oxidative damage in HaCaT cells and comprehensive analysis of key genes

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Isorhamnetin (ISO) is a methylated flavonol present in the leaves, flowers, and fruits of many plants with antitumour, anti-inflammatory, antioxidant, and anti-apoptotic properties. ISO has been suggested as the active substance in *Vernonia anthelmintica* (L.) to treat vitiligo. However, the mechanisms underlying its effects remain unclear. In this study, human keratinocytes (HaCaT cells) were pre-treated with or without ISO and then stimulated with hydrogen peroxide (H₂O₂) to generate oxidative damage. Pre-treatment with ISO increased HaCaT cell viability, reduced malondialdehyde content, and enhanced superoxide dismutase activity, resulting in a reduction in the loss of mitochondrial membrane potential, improved cell morphological damage, and apoptosis inhibition. Furthermore, we identified 51 significantly dysregulated differentially expressed genes (DEGs) of HaCaT cells treated with ISO using RNA-sequencing. Enrichment analysis using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases indicated that the protective effect of ISO could be related to its effects on the Wnt signalling pathway. Our study provides novel insights into key gene regulation in the progression of oxidative damage and the mechanisms of action of ISO.

Vitiligo is a common depigmentation disorder, clinically characterised by melanocyte destruction and melanosome loss, with a prevalence of 0.5–2% worldwide¹. Vitiligo is often accompanied by various autoimmune diseases such as hyperthyroidism, diabetes, alopecia areata, psoriasis, and asthma^{2,3}. It can occur in any part of the body, especially the exposed and wrinkled parts that are prone to friction and exposed to sunlight, and the palm plantar, mucosa, and retina can also be involved. Vitiligo affects a person's appearance and can seriously impact their work and personal relationships; patients with vitiligo often suffer from social discrimination, causing anxiety or depression and other mental disorders, as the disfiguring spots hamper their social interactions². Moreover, the disease is persistent and readily relapses; further, the treatment of this condition is difficult⁴. Although the exact cause of, or mechanism underlying, vitiligo remains mostly unknown, many theories have been proposed to explain the mechanism of pigmentation loss, such as autoimmunity, neural and genetic disease, impaired melanocyte migration and/or proliferation, and oxidative stress⁵. Oxidative stress is considered one of the most crucial initiators of vitiligo; it is involved in the development of vitiligo and is one of the factors responsible for the induction of the autoimmune response associated with the condition^{6,7}.

Oxidative stress disturbs redox homeostasis and causes an imbalance between pro-oxidants and antioxidants⁸. Oxidative stress in tissues and cells results from excessive levels of reactive oxygen species (ROS). Oxidative stress-induced damage causes excessive aggregation of hydrogen peroxide (H₂O₂) in the epidermis of vitiligo patients, and a large increase in intracellular ROS levels. Oxidative stress can further cause oxidative damage to nucleic acids, proteins, and lipids, subsequently resulting in local melanocyte damage, apoptosis, and induction of an autoimmune response to attack more melanocytes, which causes progressive skin pigment loss⁹. Accumulating evidence indicates that keratinocytes are vital for the progression of vitiligo¹⁰. Although melanocytes are

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the main target cells of vitiligo, keratinocytes are also affected by the melanocyte destruction, which interrupts a vital nexus in the mechanisms underlying vitiligo development and progression¹¹. Keratinocytes are mediators of detrimental oxidative stress, numerically dominate the cell composition of the epidermis, and directly interact with melanocytes. They can also transfer H_2O_2 to neighbouring melanocytes, which aggravates ROS accumulation and induces melanocyte destruction, thereby promoting vitiligo¹². Oxidative stress has also been shown to induce damage to keratinocytes. Melanocytes and keratinocytes constitute the epidermal melanin units, which are closely related in structure and function, and play an important role in the transmission of melanosomes¹³. Keratinocytes, as mediators of oxidative stress, secrete cytokines to recruit autoreactive T cells that interfere with signal transduction in melanocytes, subsequently inducing melanocyte destruction^{10–12}.

Isorhamnetin (ISO, 3-methyl quercetin; molecular formula: $C_{16}H_{12}O_7$) is a methylated flavonol present in the leaves, flowers, and fruits of many plants^{14,15}. ISO has numerous biological activities, including antitumour, anti-inflammatory, antioxidant, and anti-apoptotic properties^{16–18}. Increasing evidence indicates that ISO from the plant material of *Schisandra rubriflora*, and its phenolic and anthocyanin compounds, has high antioxidant potential^{19,20}. Moreover, the presence of phenolic compounds in bee bread significantly reduced the levels of oxidised-low-density lipoprotein (oxLDL) and malondialdehyde (MDA). It also significantly increased aortic antioxidant activities such as those of superoxide dismutase (SOD) and glutathione peroxidase (GPx)²¹. In addition, recent findings indicate that ISO is the main active substance in *Vernonia anthelmintica* (L.), and significantly increases the expression of melanin-biosynthetic genes (*MC1R*, *MITF*, *TYR*, *TYRP1*, and *DCT*) and tyrosinase activity in B16F10 cells²². *Vernonia anthelmintica* (L.), a traditional herbal medicine that is only grown in Kashgar, Aksu, and other areas in Xinjiang and India, is the most common herbal medicine for the treatment of vitiligo²³. However, its mechanism of action remains unclear owing to the complexity of multiple ingredients and the lack of research on its chemical composition. In our previous study²⁴, we identified 38 flavonoids from the seeds of *V. anthelmintica* (L.) using selective response/multiple response monitoring technology, and the content of total flavonoids was 0.97 g/100 g, among which ISO was present at a concentration of 93.23 ± 9.55 ng/g.

Currently, the pathogenesis of vitiligo is not well understood, treatment is difficult, and relapse is common²⁵. Several treatment options aim to restore pigmentation, including excimer lasers, vitamin D analogues, and steroid therapy²⁶. Unfortunately, these treatments are not widely employed because they induce long-term side effects²⁷. Thus, several studies have focused on the identification of novel therapeutic medicines and on the assessment of multiple targets to explain the complex network of vitiligo-associated mechanisms. The aim of this study was to investigate the antioxidant activity and identify key genes to help reduce H_2O_2 -induced oxidative damage in HaCaT cells (a human keratinocyte cell line) after ISO treatment. The results of the current study are expected to contribute to the development of ISO as a source of antioxidants and provide a theoretical basis for the targeted therapy of vitiligo.

Results

ISO protects HaCaT cells from H_2O_2 -triggered injury. The effects of different concentrations of H_2O_2 on the viability, MDA content, and SOD enzyme activity in HaCaT cells are shown in Fig. 1. The MDA content (Fig. 1b) was increased in a dose-dependent manner and the SOD level (Fig. 1c) was reduced significantly as the concentration of H_2O_2 increased. Cell viability (Fig. 1a) in the H_2O_2 -alone group (300, 600, 900, or 1200 μM H_2O_2) was significantly reduced compared with that of the control group (no H_2O_2), indicating that H_2O_2 induced cytotoxic damage to HaCaT cells ($p < 0.05$). The half-maximal inhibitory concentration (IC_{50}) value of H_2O_2 was approximately 600 μM . Based on the above results, 600 μM H_2O_2 treatment was selected to establish the oxidative-stress model of HaCaT cells.

The protective effect of ISO on the viability of HaCaT cells treated with H_2O_2 was detected using the CCK-8 assay. As shown in Fig. 1, as the concentration of ISO increased, the cell viability (Fig. 1d) in the experimental group (20, 40, or 60 μM ISO) became significantly higher than that in the H_2O_2 -alone group. The expression levels of MDA (Fig. 1e) were higher in the H_2O_2 -alone group than in the control group. Compared with the H_2O_2 -alone group, the level of MDA in the samples pre-treated with ISO was significantly lower. The expression level of SOD (Fig. 1f) in the H_2O_2 -alone group was significantly reduced compared with that in the control group, whereas the SOD level in cells pre-treated with ISO was significantly increased compared with that of the H_2O_2 -alone group. These results indicate that ISO pre-treatment can significantly reduce the inhibitory effect of H_2O_2 on antioxidant enzymes.

ISO protects HaCaT cells from H_2O_2 -induced apoptosis. The effect of ISO on the morphology of HaCaT cells injured by H_2O_2 is shown in Fig. 2. Compared with the control group (Fig. 2a), treatment of HaCaT cells with 600 μM H_2O_2 (Fig. 2b) induced apoptosis; compared with the H_2O_2 -alone group, cells pre-treated with 40 μM ISO (Fig. 2c) revealed improved cell morphology and a reduction in the number of apoptotic cells. Apoptosis is mediated via mitochondrial membrane permeabilisation events¹⁸. To investigate whether ISO can inhibit H_2O_2 -induced apoptosis stemming from mitochondrial dysfunction, the effect of ISO on the mitochondrial membrane potential of HaCaT cells after H_2O_2 -triggered injury was assessed. Confocal microscopy showed that the mitochondria of H_2O_2 -induced cells emitted weak red fluorescence and strong green fluorescence (Fig. 2d), indicating that the mitochondrial membrane potential of cells was disrupted. However, after ISO pre-treatment, the red fluorescence was significantly higher than that in the H_2O_2 -alone group (Fig. 2e), indicating that the increase in green fluorescence intensity was almost completely prevented by ISO. These results showed that ISO could reduce the loss of mitochondrial membrane potential in HaCaT cells with H_2O_2 -triggered injury.

To investigate the effects of ISO on the H_2O_2 -induced apoptosis of HaCaT cells, propidium iodide (PI) staining was performed, as shown in Fig. 2f. The apoptosis rates of the control group, H_2O_2 -alone group, H_2O_2 + 20 μM ISO, H_2O_2 + 40 μM ISO and H_2O_2 + 60 μM ISO were $4.13 \pm 0.21\%$, $20.07 \pm 0.85\%$, $15.17 \pm 0.55\%$, $8.07 \pm 0.15\%$

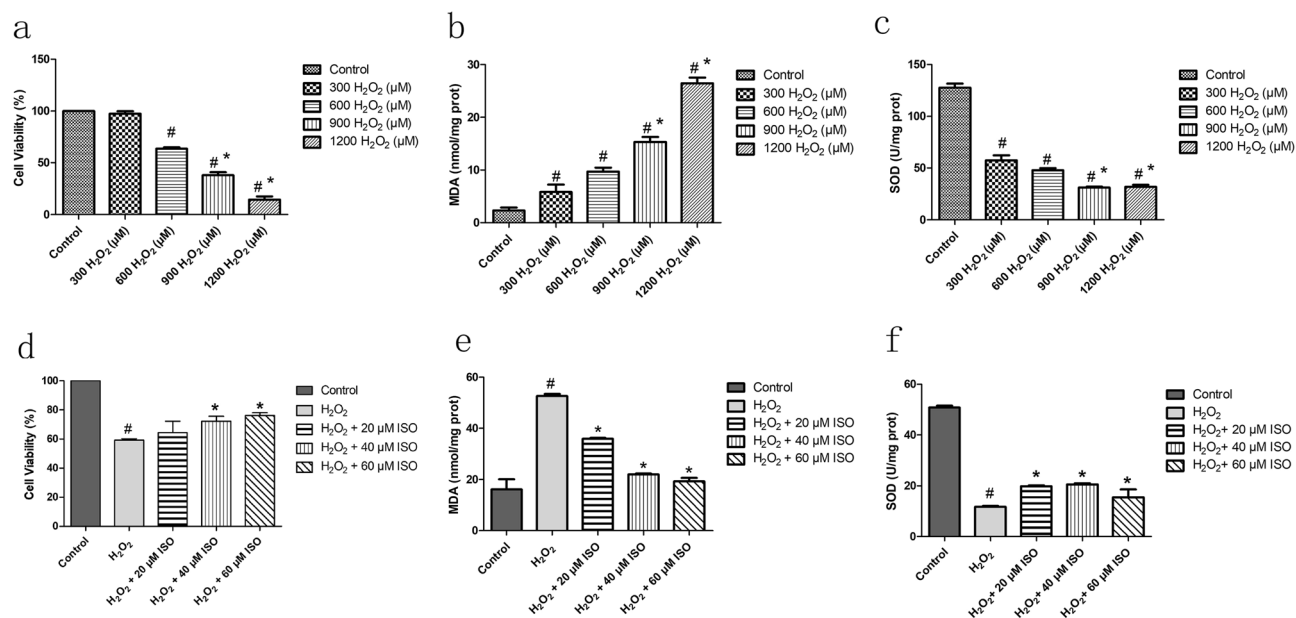


Figure 1. Effect of ISO on the cell viability, MDA content, and SOD enzyme activity after H_2O_2 -treatment. The effect of H_2O_2 on (a) the cell viability and (b) MDA content and (c) SOD enzyme activity in HaCaT cells. Control, untreated HaCaT cells; The experimental groups, cells were treated with a concentration 300, 600, 900, 1200 $\mu\text{mol/L}$ of the experimental sample for 24 h. The effect of ISO on (d) the cell viability, (e) MDA content, and (f) SOD enzyme activity in HaCaT cells. Control, untreated HaCaT cells; H_2O_2 -alone group, HaCaT cells treated with 600 μM H_2O_2 ; all H_2O_2 + cells were treated with ISO 600 μM H_2O_2 . Graphs show the mean $\pm$ SD values of three replication. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. # indicates significant differences ($p < 0.05$) from the control group. * indicates significant differences ($p < 0.05$) from the treatment of HaCaT cells with 600 μM H_2O_2 group.

and $7.13 \pm 0.21\%$, respectively. Thus, compared with the H_2O_2 -alone group, cell apoptosis was alleviated in the groups pre-treated with ISO, in a dose-dependent manner.

Differentially expressed genes (DEGs) and functional enrichment analysis. We selected three groups of HaCaT cells (control, H_2O_2 -alone, and H_2O_2 + 40 μM ISO), and used three biological replicates for RNA sequencing (RNA-seq). DEGs were identified according to the criteria of $p < 0.05$ and $|\log_2 \text{FC}| > 1$ as a measure of significance for DEGs. Volcano plots (Fig. 3a,b) show the DEG expression profiles of the H_2O_2 -alone vs. control groups and, the ISO group vs. H_2O_2 -alone group. A total of 2316 DEGs (906 upregulated and 1410 downregulated) in the H_2O_2 -alone vs. control groups, and 262 DEGs (147 upregulated and 115 downregulated) in the ISO vs. H_2O_2 groups were identified.

A Venn diagram of the identified DEGs is shown in Fig. 3c–e. There were 51 common DEGs between the H_2O_2 vs. control and H_2O_2 vs. ISO comparison groups (Fig. 3c). Twenty-three genes were upregulated by H_2O_2 and downregulated by H_2O_2 + ISO (Fig. 3d), including follistatin and glycerol-3-phosphate acyltransferase 3 (Fig. 4a). In addition, 28 genes were downregulated by H_2O_2 and upregulated by H_2O_2 + ISO (Fig. 3e), including keratin 3 (KRT3) and Wnt family member 4 (WNT4) (Fig. 4b). Treatment with ISO reversed the effect of H_2O_2 , producing gene expression profiles similar to those observed in the control group. We subsequently performed a functional enrichment analysis using these 51 DEGs. A heat map (Fig. 4c,d) depicts selected DEGs.

To gain a better understanding of the potential relationships among these 51 DEGs, we conducted a functional enrichment analysis. Gene Ontology (GO) analysis (Fig. 4a) revealed that the biological processes of the top 30 DEGs, as shown in Fig. 5a, and the most enriched biological processes (BP) were blood vessel development, vasculature development, and tertiary alcohol metabolic process. The most enriched cellular component (CC) terms were receptor regulator activity, receptor ligand activity, and phenanthrene 9,10-monooxygenase activity, and the most enriched molecular function (MF) terms were extracellular region, Golgi lumen, and extracellular region. The top enriched KEGG pathways are shown in Fig. 5b, with the bubble diagram illustrating the top 20 enriched pathways, suggesting that the Wnt signalling pathway; steroid hormone biosynthesis; and neomycin, kanamycin, and gentamicin biosynthesis may be involved in the protective effect of ISO against oxidative stress in HaCaT cells.

DEG interaction network analysis. We applied the interaction relations in the STRING protein interaction database (<http://string-db.org/>), and directly extracted the interaction relations of target DEG sets from the database to construct the network in the database. The interaction network diagram of DEGs shows that for the control vs. H_2O_2 -alone group comparison, the upregulated DEGs protein phosphatase 2 scaffold subunit A beta, signal transducer and activator of transcription 1, and cytoplasmic linker associated protein 2, and the down-

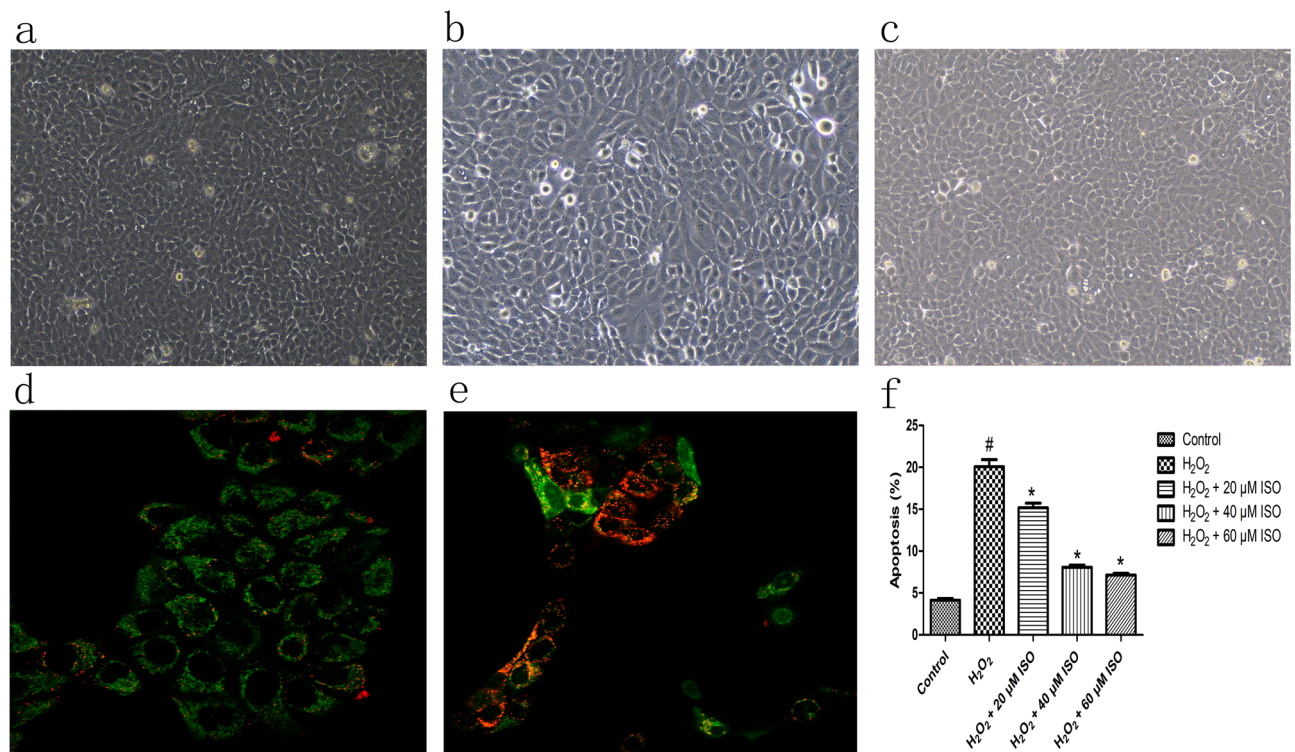


Figure 2. The effect of isorhamnetin (ISO) on the morphology of HaCaT cells injured by H₂O₂ (40× magnification). (a) Untreated HaCaT cells showing a normal cell morphology. (b) HaCaT cells stimulated with 600 μM H₂O₂ showed increased apoptosis. (c) HaCaT cells treated with 600 μM H₂O₂ + 40 μM ISO group, compared with H₂O₂-only treatment, showing improvement in cell morphology and a reduction in the number of apoptotic cells. (d) Confocal microscopy images of HaCaT cells were treated with 600 μM H₂O₂ to detect mitochondrial permeability; weak red fluorescence and strong green fluorescence indicates disruption of mitochondrial membrane potential. (e) Confocal microscopy of cells treated with 600 μM H₂O₂ + 40 μM ISO treatment, showing stronger red fluorescence. (f) Apoptosis rate of cells treated with hydrogen peroxide as assessed by PI staining and flow cytometry. Control, untreated HaCaT cells; H₂O₂-alone group, HaCaT cells treated with 600 μM H₂O₂; all H₂O₂ + ISO were 600 μM H₂O₂. Graphs show the mean ± SD values of three replication. One-way ANOVA followed by Tukey's post-hoc test. # indicates significant differences ($p < 0.05$) from the control group. * indicates significant differences ($p < 0.05$) from the treatment of HaCaT cells with 600 μM H₂O₂ group.

regulated DEGs mitogen-activated protein kinase 3, adenylate cyclase 6, and histone deacetylase 5 were located in the centre of the network, suggesting that these may be the key genes involved in the oxidative stress-induced damage of HaCaT cells.

Discussion

Vernonia anthelmintica (L.) Willd has been demonstrated to possess several pharmacological properties, including anti-inflammatory, antibacterial, antioxidant, hypoglycaemic, and antithrombotic activities²⁸. Its seeds are used in traditional therapy for the treatment of skin diseases, including vitiligo²⁹, and its chemical composition is complex; the effective components for vitiligo treatment are mainly flavonoids and caffeic acids³⁰. However, current research on vitiligo treatment with *Vernonia anthelmintica* (L.) Willd is mostly limited to the total flavonoid extraction level, and there are few studies on flavonoid monomers. In our previous study, we identified 38 flavonoids from the seeds of *V. anthelmintica* (L.) that contain ISO, a bioflavonoid with potential antioxidant properties¹⁵. To the best of our knowledge, this is the first comprehensive study to reveal the key genes involved in the protective effect of ISO against H₂O₂-induced oxidative damage in vitro. Our findings indicated that pre-treatment with ISO markedly increased cell viability in a dose-dependent manner, more than was observed for cells treated with H₂O₂ alone. We also found that pre-treatment with ISO reduced the MDA content, enhanced SOD activity, and inhibited apoptosis. Mitochondrial dysfunction is considered an important contributing factor to a variety of pathophysiological situations including aging, heart ischemia/reperfusion injury, diabetes, and cardiovascular diseases, all of which are associated with cell death^{31,32}. Mitochondrial dysfunction results in the collapse of the mitochondrial membrane potential, and it has also been shown that the mitochondrial membrane potential decreases in the early stage when stimulated by oxidative stress, and the damage can be reversed by antioxidant treatment³³. In our study, we found that pre-treatment with ISO reduced the loss of mitochondrial membrane potential, improved cell morphological damage, and inhibited apoptosis in H₂O₂-treated HaCaT cells. This indicates that ISO reverses the mitochondrial damage to some extent and sheds new light on the potential of ISO to serve as an antioxidant therapy for vitiligo.

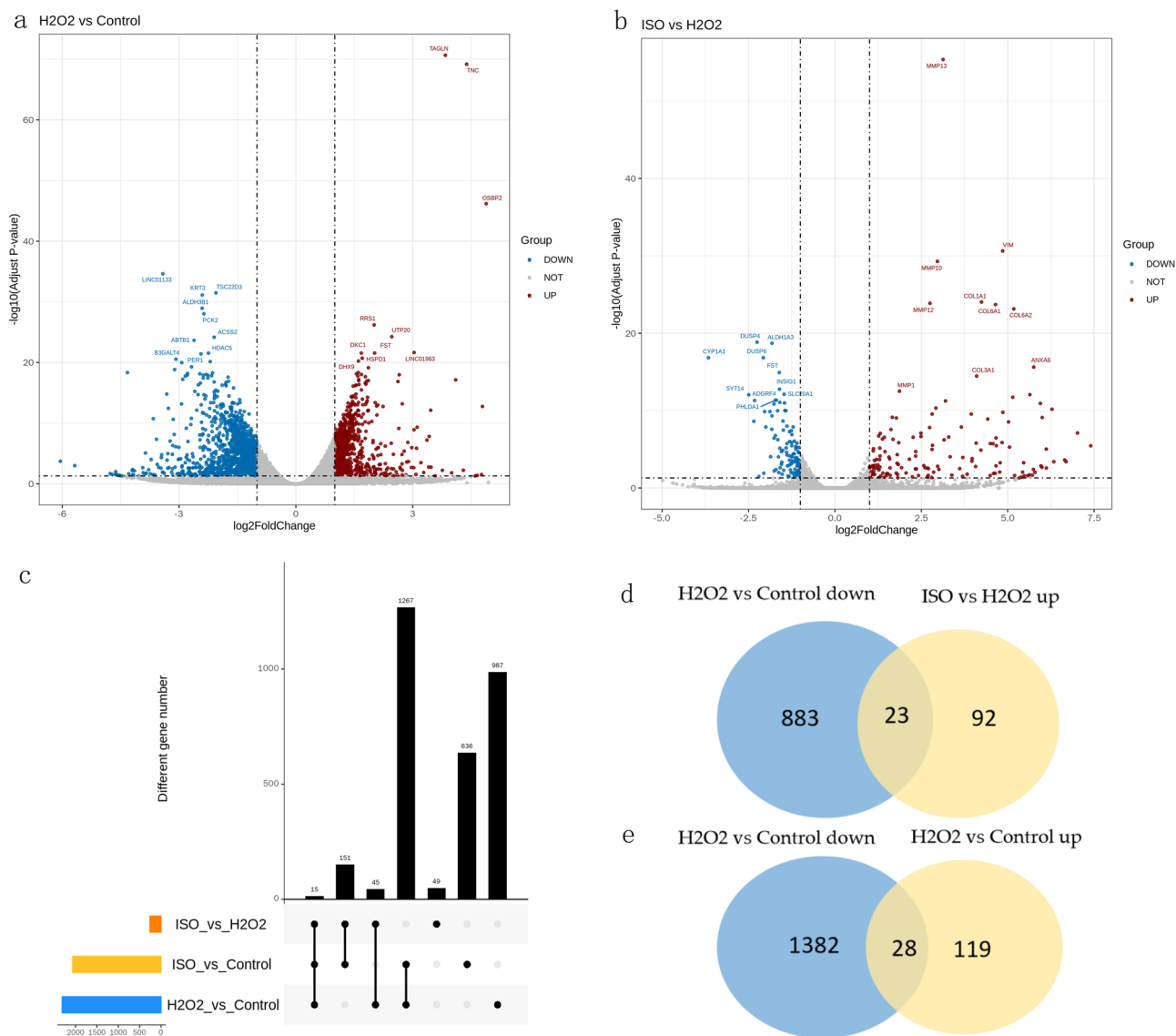


Figure 3. Differentially expressed genes (DEGs). **(a,b)** Volcano plot of gene expression profiles in the **(a)** control vs. H₂O₂ and **(b)** ISO vs. H₂O₂ comparisons. **(c–e)** Venn diagrams showing **(c)** unique or shared DEGs for those **(d)** upregulated in H₂O₂ vs. control and downregulated in H₂O₂ + ISO and genes that are **(e)** downregulated in H₂O₂ vs. control and upregulated in H₂O₂ + ISO. H₂O₂ hydrogen peroxide, ISO isorhamnetin, control group untreated HaCaT cells, H₂O₂ group HaCaT cells treated with 600 μ M H₂O₂ for 12 h, ISO group 600 μ M H₂O₂ + 40 μ M ISO.

We focused on the DEGs affected in both H₂O₂-induced HaCaT cells and those pre-treated with ISO. The DEGs observed in our model system of oxidative stress could be helpful for identifying specific biomarkers of oxidative stress-related diseases. In our study, a total of 2316 H₂O₂-responsive genes (906 upregulated and 1410 downregulated), and 262 ISO-responsive genes (147 upregulated and 115 downregulated) were detected, and a large number of DEGs were functionally characterised. By comparing the gene expression of the three groups, we determined that 51 DEGs were potentially related to the protective effect of ISO against oxidative stress in HaCaT cells. Of these, three genes were newly discovered, thus, further follow-up studies are warranted for their functional annotation. Regarding the other genes that have been previously annotated, some are involved in oxidative stress: follistatin protects against oxidative stress and apoptosis both in vitro and in vivo in chronic kidney disease³⁴. Moreover, interleukin-1 receptor antagonist (*IL-1RA*) was shown to ameliorate pain hypersensitivity, spinal inflammation, and oxidative stress induced by systemic lipopolysaccharides in neonatal rats³⁵. *CTRP3* ameliorates fructose-induced metabolic-associated fatty liver disease by inhibiting xanthine oxidase-associated oxidative stress³⁶. Some of the identified genes are also involved in apoptosis. Radiation-induced apoptosis enhancing nuclease (*AEN*) cleaves DNA in concert with other apoptotic nucleases, thereby enhancing apoptosis following ionising irradiation³⁷, and knockdown of the MET transcriptional regulator *MACC1* sensitises cancer cells to death receptor-mediated apoptosis in solid cancers³⁸. Similar results were observed in the present study.

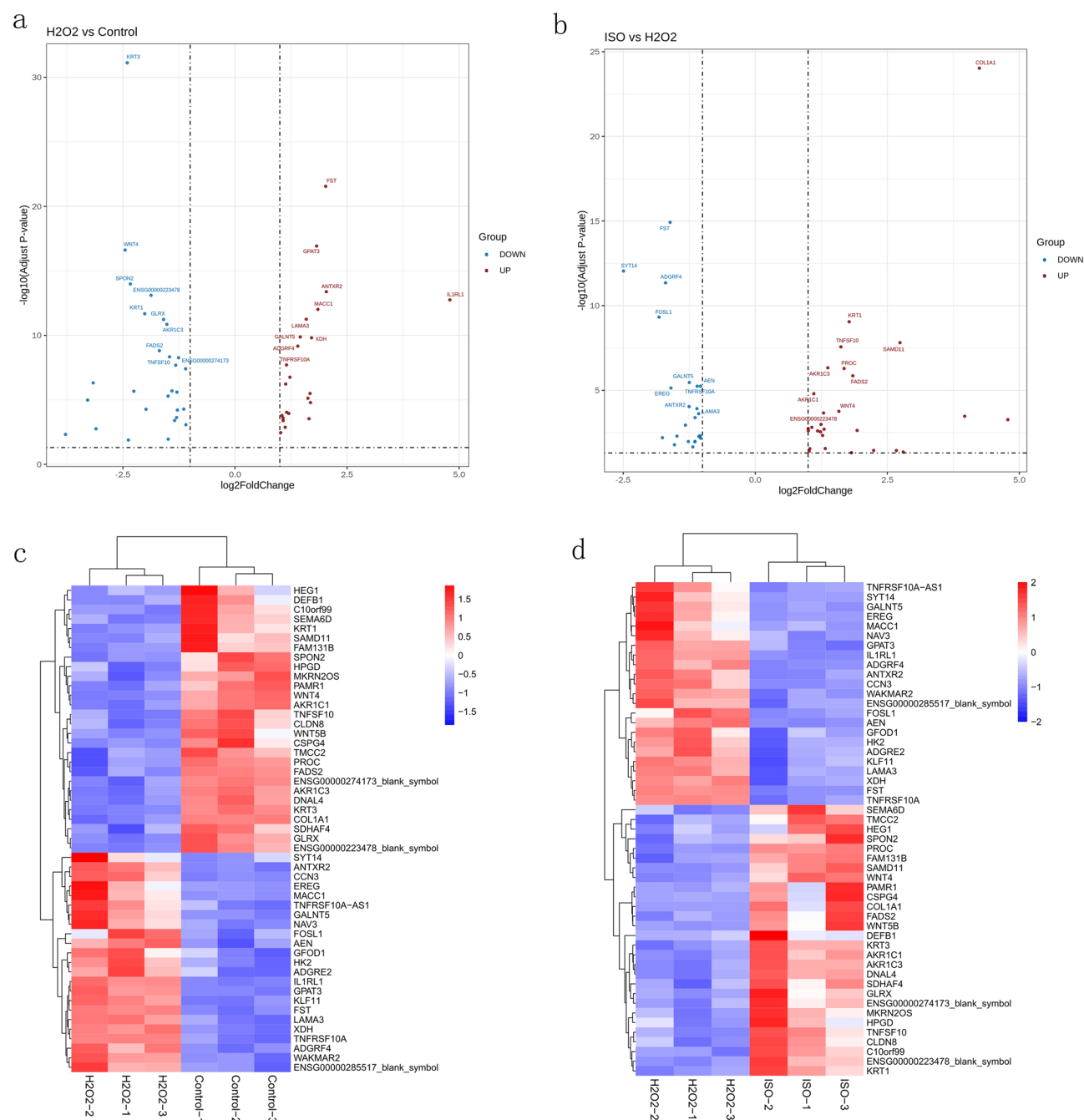


Figure 4. Functional analysis. (a,b) Volcano plot and (c,d) Heatmaps of the (a,c) 23 DEGs that were upregulated by H_2O_2 and downregulated by ISO and the (b,d) 28 DEGs that were downregulated by H_2O_2 and upregulated by ISO. The heatmap was generated by TB tools software. H_2O_2 hydrogen peroxide, *ISO* isorhamnetin, *control group* untreated HaCaT cells, H_2O_2 group HaCaT cells treated with 600 μM H_2O_2 for 12 h, *ISO group* 600 μM H_2O_2 + 40 μM ISO.

Elevated levels of *FST*, *IL1RL1*, *XDH*, *AEN*, and *MACC1* were identified as DEGs in the H_2O_2 -treated group compared to the control group. Notably, the expression of these genes decreased after pre-treatment with ISO. However, there is limited knowledge regarding the potential functions of these dysregulated DEGs. Future studies will contribute to identifying the precise mechanism of these DEGs in the protective effects of ISO against oxidative injury.

Moreover, 51 DEGs were identified as being possibly related to the antioxidant activities of ISO, and the KEGG enrichment function analysis suggested that the protective effect of ISO may be related to the Wnt signalling pathway and steroid hormone biosynthesis. Wnt signalling plays a crucial role in melanocyte stem cell differentiation³⁹. Moreover, it has been shown that Wnt/ β -catenin signalling plays a pivotal role in cell proliferation, migration, and differentiation in vitiligo pigmentation systems⁴⁰. Regazzetti et al.⁴¹ demonstrated that

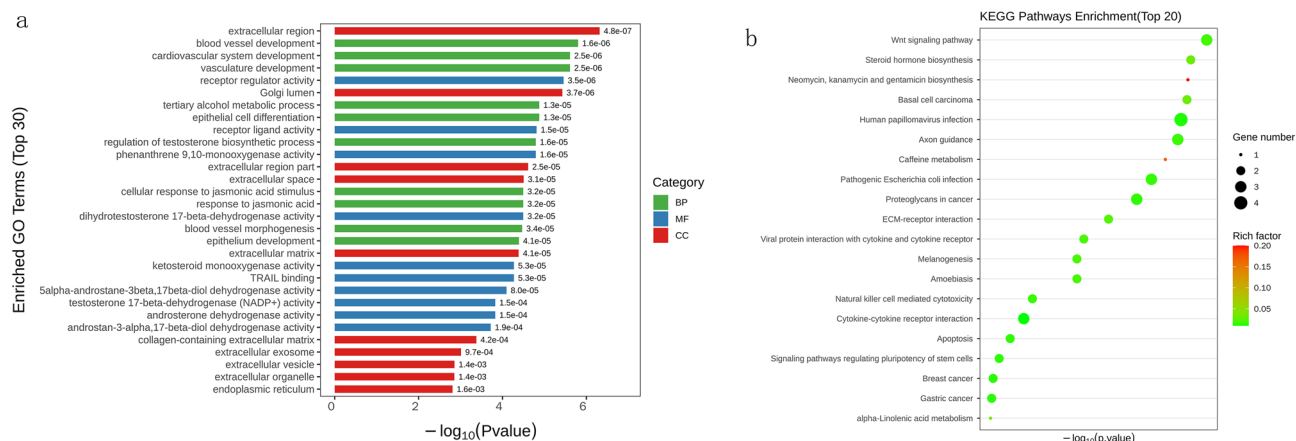


Figure 5. (a) Gene Ontology analysis of differentially expressed genes (DEGs) altered by isorhamnetin (ISO). The y-axis shows the GO term, and the x-axis shows the $-\log_{10}(p\text{ value})$. (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment bubble map of DEGs altered by ISO. The y-axis shows the pathway name, and the x-axis shows the corresponding $p\text{ value}$ of the pathway. The rich factor is the number of DEGs/the total number of genes detected in the pathway.

in lesional vitiligo, oxidative stress decreases Wnt expression in melanocytes and keratinocytes. They further demonstrated that pharmacological agents that activate the Wnt pathway successfully induced the differentiation of resident stem cells into pre-melanocytes, suggesting that stimulation of Wnt signalling may serve as an adjunct to current therapies for vitiligo⁴². Mei et al. found that the Wnt5a gene in the canonical Wnt/ β -catenin pathway can promote melanocyte differentiation and proliferation⁴³. The present study showed that ISO might exert an antioxidant effect by influencing the Wnt pathway, with involvement of Wnt family DEGs (*WNT4* and *WNT5B*). These observations may support further clinical exploration of Wnt agonists to repigment vitiligo lesions. Based on data from public databases, the DEG network was visualised for a comprehensive analysis of key genes and gene pathways involved in the counteraction of H_2O_2 -induced oxidative damage in HaCaT cells by ISO. This approach could suggest new targeted therapies for vitiligo. Furthermore, GO analysis revealed that the most enriched biological process, molecular function, and cellular component terms of these DEGs were the tertiary alcohol metabolic process, receptor regulator activity, and extracellular region, respectively. DEG interaction network analysis suggested that the key genes were closely involved in the oxidative stress damage in HaCaT cells. Future studies are likely to characterise the mechanisms of action of new transcripts and genes in the protective effects of ISO against oxidative injury.

The present investigation had some limitations. First, validation experiments should be conducted to determine whether manipulating the expression of the top candidate DEGs can affect the various functional readouts of ISO-treated and H_2O_2 -induced HaCaT cells. Further research is necessary to observe potential changes in the apoptotic rate of H_2O_2 -induced HaCaT cells by manipulating the expression of top DEG candidates. Additionally, DEG changes in animal models of oxidative damage should also be examined. Second, to address the lack of research on the effect of ISO-involved antioxidant pathways, future studies should aim to more accurately demonstrate the antioxidant stress mechanism of ISO. Third, although the protective effect of ISO against H_2O_2 -induced oxidative damage in HaCaT cells was investigated in the present study, further experiments and collaborative discussions should be held to identify the effect of ISO on H_2O_2 -induced melanocytes, which would improve the development of ISO as a source of antioxidants and provide a theoretical basis for the targeted therapy of vitiligo.

Conclusions

We demonstrated the protective effect of ISO against H_2O_2 -induced oxidative damage in HaCaT cells, including increased cell viability, reduced MDA content, and enhanced SOD activity compared with cells treated with H_2O_2 alone. In addition, we found that pre-treatment with ISO reduced the loss of mitochondrial membrane potential, improved cell morphological damage, and inhibited apoptosis in H_2O_2 -induced HaCaT cells. In addition, changes in the key genes of untreated HaCaT cells vs. H_2O_2 -induced HaCaT cells with and without ISO intervention were identified. By combining the functional results with those obtained from the bioinformatics analysis, this study highlighted some aberrantly expressed DEGs and gene pathways that might be related to oxidative stress and the antioxidant effect of ISO. One pathway of note was the Wnt signalling pathway.

Methods

Chemicals and reagents. H_2O_2 solution, RIPA lysis buffer, 3,5-dimethylphenol (DMSO), and phosphate-buffered saline (PBS) were obtained from Sigma (Shanghai, China). ISO ($\text{C}_{16}\text{H}_{12}\text{O}_7$), SOD, MDA, and proliferation and cytotoxicity assay (CCK-8) kits were obtained from Solarbio (Beijing, China). Dulbecco's modified Eagle medium (DMEM) and foetal bovine serum (FBS) were purchased from BI Company (Ridgefield CT, USA). Penicillin-streptomycin solution was obtained from Hyclone (Logan, UT, USA). Trypticase was purchased from Gibco (Waltham, MA, USA). Trizol was purchased from Magen; trichloromethane and isopro-

panol were purchased from Sinopharm; and the RNA Nano 6000 Assay Kit, NEBNext Ultra™ RNA Library Prep Kit for Illumina, and AMPure XP system were from Agilent Technologies (Santa Clara, CA, USA), ABclonal (Woburn, MA, USA), and Beckman Coulter (Brea, CA, USA), respectively. The BCA Protein Quantitation Kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture and treatment. Human keratinocytes (HaCaT; Kunming Cell Bank, Chinese Academy of Sciences) were cultured in DMEM supplemented with 10% FBS and a 1% antibiotic mixture containing penicillin and streptomycin. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂. The experiments were performed 24 h after the cells were seeded. HaCaT cells (8 × 10⁴ cells/well) were seeded in 96-well plates for 12 h and then treated with various concentrations (300, 600, 900, or 1200 μM) of H₂O₂. Similarly, HaCaT cells were pre-treated with various concentrations (20, 40, 60, or 80 μM) of ISO in the medium prepared above for 12 h to determine the optimal treatment concentration. Subsequently, the pre-treated cells were cultured for an additional 12 h with 600 μM H₂O₂ to stimulate oxidative damage. Three replicates per group were used for all experiments. Untreated cells and cells treated with H₂O₂ alone were used as controls.

Cell viability assay. The cellular toxicity of H₂O₂ and the protective effect of ISO were measured in HaCaT cells using the CCK-8 assay. After treatment with the indicated concentrations of H₂O₂ or ISO, 10 μL of the CCK-8 kit solution was added, and the cells were incubated for 2 h at 37 °C. Subsequently, the absorbance was measured using a microplate reader (Thermo Fisher Scientific).

Biochemical analysis. Cold PBS was used to collect treated and untreated HaCaT cell samples after centrifugation at 157×g for 3 min according to the instructions provided in each enzyme kit. The cells were homogenised with cold PBS, and commercial kits were used to obtain the supernatant for the measurement of MDA and SOD activities.

Cell apoptosis assay. The protective effect of ISO on the oxidative stress-induced apoptosis of HaCaT cells was determined using the Hoechst assay. Cells pre-treated with 20, 40, and 60 μM ISO were used as the experimental group. After treatment of the cells, 5 μg/mL Hoechst staining solution was added, and the cells were incubated for 10–15 min at 25 °C in the dark. Subsequently, 400 μL of 1 × binding buffer was added, mixed, and placed on ice, and the images of the cells were captured within 1 h under an Olympus fluorescence microscope. This experiment was repeated three times.

SOD activity. SOD activity in HaCaT cells was measured using the nitroblue tetrazolium method. HaCaT cells at the logarithmic growth stage were digested and washed. The cell suspension was centrifuged at 157×g for 5 min. HaCaT cells (1 × 10⁶ cells/well) were seeded in 6-well plates for 12 h and grown at 37 °C in a humidified atmosphere containing 5% CO₂. When the cells adhered to the wells and the density was approximately 80%, the cells were treated with various concentrations (20, 40, or 60 μM) of ISO for 12 h. Subsequently, the pre-treated cells were cultured for an additional 12 h with 600 μM H₂O₂ for 12 h. The cell suspension was then mixed thoroughly with a vortex mixer (ice bath; power 20% or 200 W; ultrasound 3-s, 10-s intervals, 30 repeats) and centrifuged at 8000×g at 4 °C for 10 min. The reagents were added to the cell suspension, according to the manufacturer's instructions. Finally, the mixture was thoroughly mixed and cultured at 37 °C for 30 min, placed in a 1-mL glass cuvette, and the maximal absorbance of the mixture was measured at 560 nm using a spectrophotometer. One unit of SOD was defined as the amount of enzyme that caused 50% inhibition.

MDA assay. The MDA concentration was used to determine lipid peroxidation. The cell suspension was mixed thoroughly with a vortex mixer (ice bath; power 20% or 200 W; ultrasound 3-s, 10-s intervals, 30 repeats) and centrifuged at 8000×g at 4 °C for 10 min. The reagents were added to the cell suspension, according to the manufacturer's instructions. Finally, the mixture was thoroughly mixed and incubated at 100 °C for 60 min, cooled in an ice bath, and centrifuged at 10,000×g for 10 min. Two hundred microliters of supernatant were transferred to a microglass cuvette or 96-well plate, and the maximal absorbance was determined at 450 nm, 532 nm, and 600 nm using a spectrophotometer. The MDA content was calculated according to the protein concentration.

Mitochondrial ultrastructure observation. HaCaT cells were inoculated into 25-cm² culture flasks and cultured to approximately 80% confluency for fusion. Experimental groups were divided into four groups: blank, control, H₂O₂-alone, and ISO + H₂O₂. The treatment methods for each group were the same as those described above. Cells were collected into 10-mL centrifuge tubes 24 h after routine digestion and centrifuged at 1000×g for 5 min. The supernatant was discarded and 300 μL of high-sugar complete medium was added to the cell precipitate for resuspension. The cell suspension was transferred into a 0.5-mL EP tube, centrifuged at 2000×g for 8 min, and the supernatant was carefully removed. The EP tube was tilted at 45°, and 2.5% glutaraldehyde solution was slowly added to the tube wall with a pipette tip until the entire EP tube was filled, followed by fixation at 4 °C for 24 h. The fixed specimens were sent to the Electron Microscopy Center of the Basic Laboratory of Xinjiang Medical University for transmission electron microscopy.

RNA extraction and monitoring of quality. We selected three groups of HaCaT cells for RNA sequencing: untreated cells, 600 μM H₂O₂-induced cells, and H₂O₂-induced cells pre-treated with 40 μM ISO (pre-treatment with ISO markedly increased cell viability in a dose-dependent manner versus that observed for cells

treated with H₂O₂ alone. 40 µM ISO was significantly different to 20 µM but there was no difference with 60 µM. The cells pre-treated with 40 µM ISO were used as the protection group). Total RNA was extracted from the cells using TRIzol reagent according to the manufacturer's instructions. The absorbance ratio at 260–280 nm of the RNA samples was measured with a Nanodrop ND-2000 system (Thermo Fisher Scientific) and the RNA integrity number was determined using an Agilent Bioanalyzer 4150 system (Agilent Technologies). Only high-quality samples according to assessments of RNA purity, concentration, and integrity were used for library construction.

Library preparation and sequencing. Paired-end libraries were prepared using ABclonal mRNA-seq Lib Prep Kit (ABclonal) following the manufacturer's instructions. The mRNA was purified from 1 mg total RNA using oligo (dT) magnetic beads, followed by fragmentation using divalent cations at elevated temperatures in ABclonal First Strand Synthesis Reaction Buffer. Subsequently, first-strand cDNA was synthesised with random hexamer primers and reverse transcriptase using mRNA fragments as templates, followed by second-strand cDNA synthesis using DNA polymerase I, RNase H, and dNTPs. The synthesised double-stranded cDNA fragments were then adapter-ligated to prepare the paired-end library. Adaptor-ligated cDNA was used for polymerase chain reaction (PCR) amplification. PCR products were purified (AMPure XP system) and library quality was assessed using an Agilent Bioanalyzer 4150 system. Sequencing was performed using an Illumina Novaseq 6000/MGISEQ-T7 instrument.

Data analysis. The data generated from the Illumina/BGI platform were used for the bioinformatic analysis. All analyses were performed using an in-house pipeline from Shanghai Applied Protein Technology.

Quality control. Raw data (or raw reads) of FASTQ format were first processed using in-house Perl scripts. In this step, the adapter sequence was removed, and low-quality reads [the number of lines with a string quality value less than or equal to 25 accounting for more than 60% of the entire read, or greater than 5% Ns (unknown base)] were filtered out to obtain clean reads that could be used for subsequent analysis.

Quantification of gene expression level and differential expression analysis. Clean reads were separately aligned to the reference genome in orientation mode using HISAT2 software (<http://daehwankimlab.github.io/hisat2/>) to obtain mapped reads, which were then spliced with Stringtie software (<http://ccb.jhu.edu/software/stringtie/>). Gffcompare software (<http://ccb.jhu.edu/software/stringtie/gffcompare.shtml>) was used for comparing the obtained sequences with the reference genome GTF/GFF file to find the original unannotated transcription region, and to discover new transcripts and new genes. FeatureCounts (<http://subread.sourceforge.net/>) was used to count the read numbers mapped to each gene. The expression level in FPKM of each gene was calculated based on the length of the gene and read counts mapped to the gene. Differential expression analysis was performed using the DESeq2 package (<http://bioconductor.org/packages/release/bioc/html/DESeq2.html>); differentially expressed genes (DEGs) with $|\log_2 \text{fold change (FC)}| > 1$ and adjusted p value (Padj) < 0.05 were considered significant DEGs.

Enrichment analysis. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses (<http://www.kegg.jp/kegg/kegg1.html>) can explain the functional enrichment of DEGs and clarify the differences between samples at the gene function level⁴⁴. We used the clusterProfiler R software package for GO function enrichment and KEGG pathway enrichment analysis. When $p < 0.05$, it was considered that the GO or KEGG function was significantly enriched. Transcription factors of the DEGs were extracted directly from the AnimalTFDB (<http://bioinfo.life.hust.edu.cn/AnimalTFDB/>)/PlantTFDB (<http://planttfdb.cbi.pku.edu.cn/>) databases.

Protein–protein interaction (PPI) analysis. PPI analysis was used to evaluate whether there was an interaction between gene products and other proteins. The analysis was based on protein information corresponding to genes. The known and predicted PPIs were obtained from the STRING database (<https://www.string-db.org/>). For the gene sequences existing in the database, the networks were constructed by extracting the target gene list from the database. For gene sequences not included in the database, the target gene set sequence was first aligned with the reference sample protein sequence contained in the STRING protein interaction database with BLASTx, and then the protein interaction relationship of the reference gene sequences was used to establish an interaction network. rMATS (<http://rnaseq-mats.sourceforge.net/index.html>), a variable splicing analysis software suitable for RNA-sequencing data, was used for analysis.

Statistical analysis. SPSS (version 22.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. Statistical significance was set at $p < 0.05$. DESeq software was used to process the sequencing data. DEGs were identified according to the criteria of $p < 0.01$ and $|\log_2 \text{FC}| > 2$.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Author contributions

K.X.J. conceived, designed, and supervised the research project. H.W. and Z.J.Z. performed the experiments and prepared the manuscript. W.H.J., D.L.H. and L.J. performed the cell experiments. H.W. and G.M.M. provided comments and suggestions regarding the preparation of the manuscript. K.X.J. supervised the research project and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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