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The protective effect of EGF-activated ROS in human corneal epithelial cells by inducing mitochondrial autophagy via activation TRPM2

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Abstract

Oxidative stress is a major pathogenesis of some ocular surface diseases. Our previous study demonstrated that epidermal growth factor (EGF)-activated reactive oxygen species (ROS) could protect against human corneal epithelial cell (HCE) injury. In the present study, we aimed to explore the role and mechanisms of oxidative stress and mitochondrial autophagy in HCE cells subjected to scratch injury. CCK-8 assays, EdU assays, Western blot analysis, wound-healing assays, and flow cytometry were conducted to determine cell viability, proliferation, protein expression, cell apoptosis, and intracellular ROS levels, respectively. The results showed that EGF could promote damage repair and inhibit cell apoptosis in scratch injured HCE cells by upregulating ROS (**$p < .01$, ***$p < .001$). EGF also induced mitochondrial autophagy and alleviated mitochondrial damage. Interestingly, the combination of the mitochondrial autophagy inhibitor and mitochondrial division inhibitor 1 (MDIVI-1) with EGF could reduce cell proliferation, viability, and the ROS level (*$p < .05$, **$p < .01$, ***$p < .001$). Treatment using the ROS inhibitor *N*-acetyl-L-cysteine abrogated the increase in mitochondrial membrane potential after EGF treatment. (*$p < .05$). Taken together, these findings indicated that EGF plays an important role in HCE damage repair and could activate ROS to protect against HCE injury by inducing mitochondrial autophagy via activation of TRPM2.

KEYWORDS
epidermal growth factor, human corneal epithelial cell, mitochondrial autophagy, reactive oxygen species, TRPM2

1 | INTRODUCTION

The corneal epithelium is the outermost layer of the cornea and consists of multiple layers of squamous epithelial cells (Chi & Trinkaus-Randall, 2013). The corneal epithelial basement membrane is located between the basal epithelial cells and the corneal stroma (Torricelli, Singh, Santhiago, & Wilson, 2013). Corneal epithelial cells are non-keratinized, stratified squamous cells that form the first defense line to protect the eye from microbial infection and various environmental hazards, including the ultraviolet-induced damage (Kumar & Yu, 2006). Corneal abrasions usually heal quickly and smoothly; however, certain risk factors may delay repair, including limbal stem cell deficiency, dry eye disease, and neurotrophic keratopathy (Channa et al., 2016; Wolf et al., 2017). Epithelial defects that do not heal within 2 weeks after treatment are known as persistent epithelial defects and can lead to permanent corneal scarring, with concurrent infection or chronic inflammation

Yanan Huo and Wei Chen contributed equally to this study.

(Jeng, 2016; Liu & Kao, 2015). Therefore, the simplicity and accessibility of the corneal structure make it a good model system to study the basic tissue repair process. It is important to explore the mechanism of human corneal epithelial (HCE) cell scratch injury to maintain corneal transparency.

Oxidative stress is characterized by the generation of reactive oxygen species (ROS), which comprise the superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), and is considered to be involved in these external stresses (Cejka & Cejkova, 2015). Under normal conditions, ROS production and scavenging are balanced. The excessive production of ROS or an imbalance of ROS production and scavenging can cause oxidative stress or induce oxidative injury or damage in the human body. ROS can induce cellular injury by targeting DNA, protein, and cytolipin (Shetty et al., 2017; R. Wei, Zhang, Xie, Shen, & Chen, 2015). The ROS system is very complicated and can be divided into ROS generation and ROS degradation (Apel & Hirt, 2004; Sinha & Dabla, 2015). In our previous study, we demonstrated that epidermal growth factor (EGF) induces transient ROS generation in corneal epithelial cells and that the presence of ROS is essential for EGF-stimulated cell proliferation, adhesion, migration, and overall corneal epithelial wound healing (Huo et al., 2009). Growth factors such as EGF play an important role in cell proliferation and wound healing of HCE cells (HCE; Imanishi et al., 2000; Zelenka & Arpitha, 2008).

Transient receptor potential cation channel subfamily M member 2 (TRPM2) can also be positively regulated by Ca^{2+}, cyclic adenosine diphosphoribose, H_2O_2, and nicotinic acid adenine dinucleotide phosphate, and is negatively regulated by AMP and acidic pH (Magnone et al., 2012; Scherz-Shouval & Elazar, 2011). TRPM2-mediated Ca^{2+} influx has been implicated in several physiological and pathophysiological processes, including insulin secretion and endothelium permeability, as well as dendritic cell maturation and chemotaxis (Sumoza-Toledo & Penner, 2011). Oxidative stress could potently activate TRPM2 for Ca^{2+} influx, thus leading to cell death (Miller, 2004). Paradoxically, oxidative stress can also activate autophagy to prevent the accumulation of ROS, thereby alleviating the damage and allowing cell survival (Scherz-Shouval & Elazar, 2011). Autophagy is an essential catabolic degradation process for cell survival, which acts as an important cellular antioxidant pathway to relieve oxidative stress (Levine, Mizushima, & Virgin, 2011; Marino, Niso-Santano, Baehrecke, & Kroemer, 2014; Mizushima & Levine, 2010). When autophagy is defective, ROS accumulates, and mitochondrial damage occurs, the latter is itself a source of ROS; thus, a feedback loop is induced that increases the ROS levels. ROS are known to induce early autophagy (Filomeni, De Zio, & Cecconi, 2015); however, the details of this association are not completely understood. Little has been reported about the association between ROS and autophagy in scratch injured HCE cells. In the present study, we aimed to investigate the protective role of EGF-activated ROS against HCE cell injury by inducing mitochondria autophagy via the TRPM2 pathway.

2 | MATERIALS AND METHODS

2.1 | Human corneal epithelial cell culture

SV40-immortalized HCE cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). HCE cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 20% fetal bovine serum (FBS; GIBCO, Grand Island, NY) and 1% units/ml penicillin and 1% streptomycin (Sigma, St. Louis, MO), 5 ng/ml EGF (AF-100-15; PeproTech, Rocky Hill, NJ) and 5 mg/ml insulin in a humidified 5% CO_2 incubator at 37°C. ROS inhibitor *N*-acetyl-L-cysteine (NAC; A7250; Sigma), Fura-2-acetoxymethyl ester (Fura-2 AM; ab120873, Abcam), mitochondrial autophagy inhibitor mitochondrial division inhibitor 1 (MDIVI-1, s7162; Selleck, Huston, TX), eTRPM2 inhibitor clotrimazole (CLT, s1606; Selleck), or cyclic adenosine diphosphate ribose (cADPR, CAS 119340-53-3; Santa Cruz Biotechnology, Dallas, TX) were added when required, based on the experiment design.

2.2 | Cell viability assay

A cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to measure relative cell viability. HCE cells (5×10^3 cells/well) were seeded in 96-well plates and cultured for 24 hr. The culture medium was replaced with 10% FBS-medium containing a different drug (EGF: 5 ng/ml, MDIVI-1: 5 μM, NAC: 20 nM, cADPR: 10 μM, CLT: 20 μM). After further incubation for 48 hr, 10 μl of CCK-8 solution was added, the cells were incubated for an additional 3 hr, and then the absorbance at 450 nm was measured using an MRX II microplate reader (Dynex Technologies, Chantilly, VA). Relative cell viability was calculated as a percentage of untreated controls.

2.3 | Flow cytometry analysis

Cells were digested with 0.25% trypsin without EDTA to prepare a single-cell suspension (2×10^5). The cells were washed twice with PBS, the supernatant was discarded, 100 μl binding buffer and fluorescein isothiocyanate (FITC)-labeled Annexin-V (20 μg/ml) at 5 μl/sample were added, and the samples incubated in the dark at room temperature for 15 min. Then, propidium iodide (PI; 50 μg/ml) was added at 5 μl/sample, and the samples were incubated in the dark for 5 min. Thereafter, 400 μl of Binding Buffer was added and the samples were immediately subjected to FACScan for quantitative detection by flow cytometry (for up to 1 hr). At the same time, a blank cell tube was used to adjust the voltage, and tubes containing Annexin-V-FITC and PI, respectively, were used for compensation adjustment.

2.4 | 5-Ethynyl-2′-deoxyuridine (EdU) assay

The cell proliferation was measured using an EdU assay according to instructions of the Click-iTEdU Imaging Kit (Invitrogen,

Carlsbad, CA). In the figures showing EdU results, all scale bars indicate 50 μm

2.5 | Wound-healing assay

A wound-healing assay was used to determine the cells migration ability. HCE cells (3×10^5) were cultured in six-well plates and confluent cells were used for the in vitro scratch-wound assay. The HCE cells were separately treated with the corresponding drugs, and cultured to 80–90% confluence. A uniform scratch wound was made across the cells in culture dish using a 200-ml plastic pipette tip. The cells were washed with PBS to remove detached cells, serum-free medium was added, and the plates were incubated in a humidified 5% CO_2 incubator at 37°C. The cells were fixed and photographed after 0 and 48 hr of incubation.

2.6 | Western blot analysis

Proteins were extracted from cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) containing protease inhibitors (Sigma). The protein concentration was quantified using bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts (40 μg) of protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was blocked with Tris-buffered saline (TBS) and 0.1% Tween 20 (TBS-T) containing 5% bovine serum albumin and then incubated with primary antibodies (Abcam, Cambridge, UK; anti-BNIP3 [BCL2 interacting protein 3], anti-Parkin [Parkin RBR E3 ubiquitin protein ligase], anti-Cytochrome *c*, anti-LC3, and anti-TRPM2; diluted 1:1,000 in TBS-T) at 4°C overnight. The meme was then washed with T-BST three times and incubated with the corresponding secondary antibodies (Abcam; diluted 1:2,000 in T-BST) for 2 hr at 37°C. Finally, the immunoreactive protein bands were visualized using chemiluminescence (GE Healthcare, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase was used as internal control.

2.7 | 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining

JC-1 staining was used to assess the changes in mitochondrial membrane potential. HCE cells were seeded on 12-well plates (5×10^5 cells/well) for 12 hr, and then treated with or without EGF, or EGF combined with NAC for 48 hr to examine the mitochondrial membrane potential. The cells were harvested and washed with PBS three times and then 5 μg/ml JC-1 (Invitrogen, Paisley, UK) was added to the cells, and incubated for 20 min at room temperature. Finally, the membrane potential was measured using a fluorescence microscope (Zeiss Fluorescence Microscope, Germany). When detecting JC-1 monomer, the excitation light was set to 490 nm and the emission light was set to 530 nm; when detecting JC-1 polymer, the excitation light was set to 525 nm and the emission light was set to 590 nm.

2.8 | Intracellular ROS measurement

The level of intracellular ROS was determined using flow cytometry analysis of the fluorescent intensity of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen). In brief, HCE cells (2×10^5/well) in a six-well plate were incubated with EGF (5 ng/ml) for 24 hr. The DCFH-DA probe was diluted 1:1,000 with serum-free medium to a final concentration of 10 μM before addition to the cells. The cells in each well were then washed three times with serum-free medium, digested by pancreatic enzymes at 37°C for 3 min, and immediately subjected to ROS measurement by flow cytometry analysis using a Cytomics FC 500 MCL (Beckman Coulter, Inc., Brea, CA). The intensity of fluorescence before and after stimulation was detected in real time or at different time points using an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.9 | Determination of intracellular calcium ion concentration

Ca^{2+} in HCE cells was determined as described previously (W. J. Wei et al., 2012). In brief, HCE cells were loaded with 2 μM Fura-2 AM. in Hank's balanced salt solution (HBSS) for 30 min at room temperature in the dark. After washing twice with prewarmed HBSS, the cells were further incubated for 30 min at 37°C in the dark. Finally, after washing with prewarmed HBSS, the HCE cells were viewed under an Olympus inverted epifluorescence microscope and fluorescence images were acquired under a confocal microscope (using the same exposure settings, Zeiss LSM 800 [Carl Zeiss]): excitation laser = 405 nm and emission gate center = 519 nm.

2.10 | Mitochondrial activity

The mitochondrial activity was examined by mitophagy detection kit (Dojindo) according to the manufacturer's instructions.

2.11 | Statistical analysis

All experimental data were presented as mean ± standard deviation and used Prism 5 (GraphPad Software, La Jolla, CA) to analyze the results. Statistical differences between two groups were examined with Student's *t* test; multiple groups were compared using one-way analysis of variance, followed by Bonferroni's post hoc test. $p \leq .05$ is considered statistical significance.

3 | RESULTS

3.1 | EGF promotes damage repair and inhibits cell apoptosis in HCE by upregulating ROS production

A previous study demonstrated that EGF stimulation could enhance HCE cell proliferation and migration via ROS signaling (Huo, Chen, & Zheng, 2015). To explore the effect of EGF in scratch injury-induced HCE cells damage, we examined the ROS levels, cell viability, and cell proliferation following the treatment with or without EGF in scratch injured HCE cells using flow cytometry, CCK-8, and EdU assays, respectively. The results showed that EGF upregulated the ROS levels in scratch injured HCE cells (*$p < .05$, ***$p < .001$; Figure 1a,b). As shown by CCK-8 and EdU assays, EGF treatment increased HCE cell viability and cell proliferation (**$p < .01$, ***$p < .001$; Figure 1c–e). Next, we assessed the effect of EGF on HCE cell migration after scratch injury using a wound-healing assay. The results showed that under the same conditions, the rate of cell migration after EGF stimulation was faster than that of the control HCE cells without EGF (*$p < .05$; Figure 1f,g). Flow cytometry showed that the treatment with EGF could reduce the increase in cell apoptosis caused by scratch injury (**$p < .01$; ***$p < .001$; Figure 1h,i).

3.2 | EGF could induce mitochondrial autophagy and alleviate mitochondrial damage

Next, we explored the underlying mechanisms of EGF in HCE cells after scratch injury. Mitochondrial autophagy plays an important role in cell damage and ROS is closely related to mitochondrial autophagy (Yin et al., 2018). Therefore, we hypothesized that mitochondrial autophagy plays a vital role in ROS-protected cell damage. We detected the changes of autophagy-related protein (BNIP3, Parkin, Cytochrome *c*, LC3I, and LC3II) after treatment with or without EGF in scratch injured HCE cells using Western blot analysis. As shown in Figure 2a–c, the levels of BPIN3, Parkin, and the ratio of LC3II:LC3I were upregulated following EGF stimulation, while Cytochrome *c* levels decreased. The intensity of mitochondrial fluorescence was increased by EGF stimulation compared with control under scratch injury(*$p < .05$, **$p < .01$; Figure 2d,e). The JC-1 monomer was increased (the mitochondrial membrane potential was reduced) after scratch injury, while it was significantly reduced after EGF treatment (*$p < .05$, ***$p < .001$; Figure 2f,g). These results revealed that the treatment with EGF could induce mitochondrial autophagy and reduce mitochondrial damage in HCE cells.

3.3 | EGF could play an important role in inducing mitochondrial autophagy

It has been reported that mitophagy inhibitors Mdivi-1 could inhibit proliferation, invasion, and metastasis of breast cancer and ovarian cancer (J. Wang, Hansen, Edwards, Van Houten, & Qian, 2015; Zhao et al., 2013). Furthermore, it could determine the relationship between mitophagy and proliferation(Niu et al., 2016).To determine if EGF indeed plays a role by inducing mitochondrial autophagy, we used MDIVI-1 (5 μM) to block the occurrence of mitochondrial autophagy and then observe whether the protective role of EGF remained. Wound-healing assays indicated that MDIVI-1 combined with EGF reduced cell mobility compared with that in the EGF group (*$p < .05$, **$p < .001$; Figure 3a,b). Furthermore, MDIVI-1 combined with EGF decreased the increase in the fluorescence intensity of mitophagy and apoptotic cells after treatment with EGF (*$p < .05$, **$p < .01$, ***$p < .001$; Figure 3c,d). EdU and CCK-8 assays also demonstrated that MDIVI-1 with EGF could reduce cell proliferation and cell viability compared with EGF group (*$p < .05$, **$p < .01$, ***$p < .001$; Figure 3e–g). The level of ROS was increased by EGF treatment, but decreased in cells treated with MDIVI-1 and EGF (*$p < .05$, ***$p < .001$; Figure 3h,i).

3.4 | The effect of EGF on cell damage and the induction of mitochondrial autophagy disappeared after treatment with the ROS inhibitor NAC

After treatment with the ROS inhibitor NAC followed by EGF, cell proliferation and cell viability were reduced (*$p < .05$, **$p < .01$, ***$p < .001$; Figures 4a,b and 4g). The cell migration ability was also reduced following treatment with NAC combined with EGF, compared with EGF alone (*$p < .05$, **$p < .01$; Figure 4c,d). The fluorescence intensity of mitophagy and apoptotic cells also decreased in the NAC + EGF group (*$p < .05$, **$p < .01$, ***$p < .001$; Figure 4e,f). Flow cytometry analysis for the ROS levels indicated that EGF combined with NAC could abrogate the increase in ROS induced by EGF treatment (**$p < .01$; Figure 4h,i). NAC combined with EGF could ameliorate the decrease in JC-1 monomer (increase in the mitochondrial membrane potential) after EGF treatment (*$p < .05$, **$p < .01$, ***$p < .001$; Figure 4j,k). These results revealed that the protective effect of EGF on cell damage and the induction of mitochondrial autophagy were abolished after simultaneous treatment with NAC.

3.5 | The expression of TRPM2 and the concentration of Ca^{2+} were increased after treatment with EGF

The TRPM2-Ca^{2+} signaling pathway plays a vital role in autophagy regulation (Q. Wang et al., 2016). We wondered whether EGF-induced upregulation of ROS was related with mitochondrial autophagy in HEC cells. Therefore, we detected TRPM2 protein levels after treatment with EGF and in untreated HCE cells and found that EGF significantly upregulated TRPM2 levels in scratch injured HCE cells (**$p < .01$, ***$p < .001$; Figure 5a,b). We next examined the concentration of TRPM2-mediated Ca^{2+} and mitochondrial apoptosis after treatment with or without EGF in scratch injured HCE cells. The results showed that EGF treatment could increase the fluorescence intensity of mitophagy and Fura-2 AM, which had decreased after scratch injury (**$p < .01$, ***$p < .001$; Figure 5c,d).

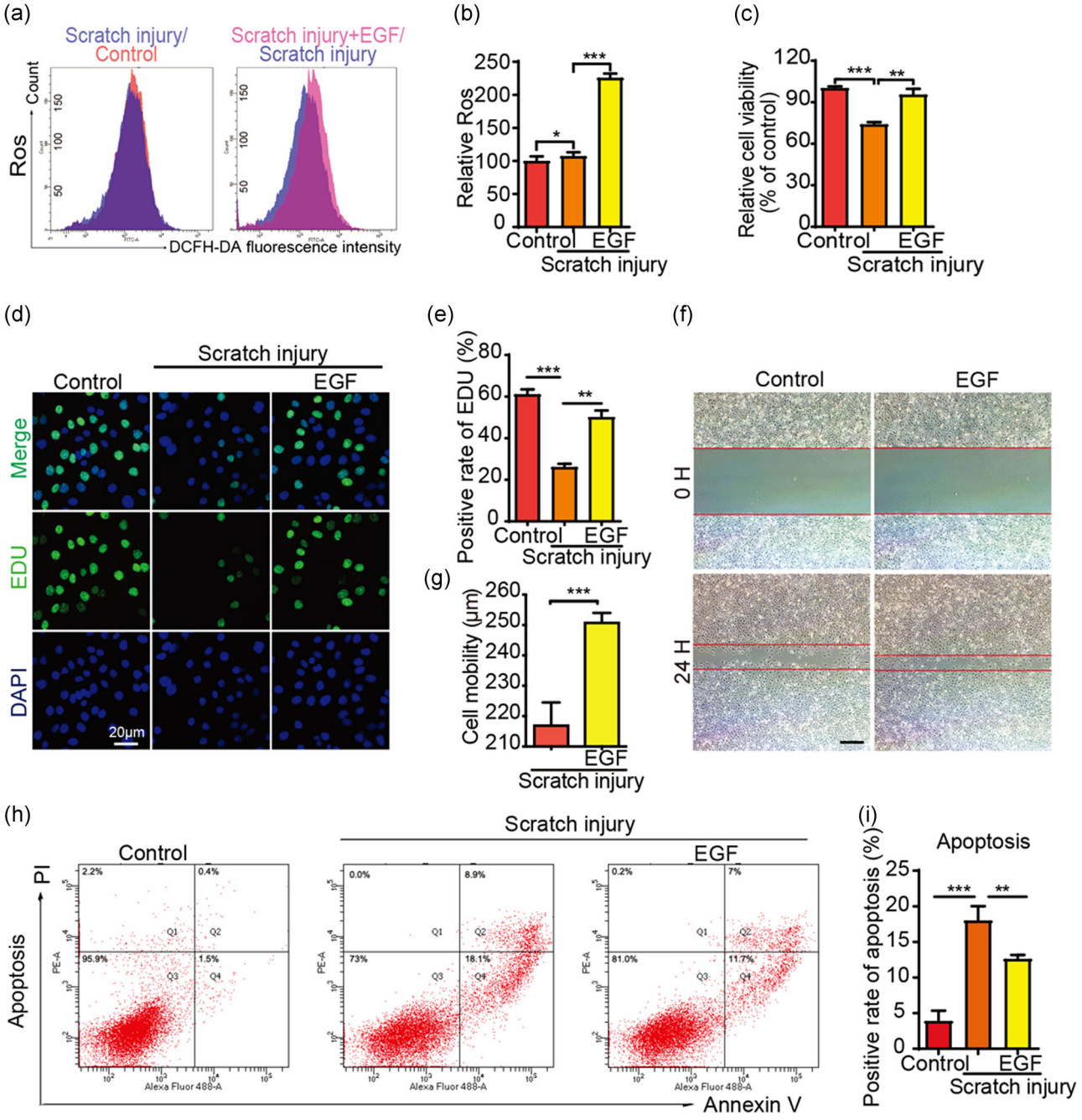

FIGURE 1 EGF promotes damage repair and inhibits cell apoptosis in HCE by upregulating ROS production. (a and b) The ROS levels were determined using flow cytometry and the results showed that EGF could increase these levels in HCE cells. *$p < .05$, ***$p < .001$. (c) CCK-8 assay of HCE cell viability in the presence of EGF after scratch injury. **$p < .01$, ***$p < .001$. (d and e) EdU assay of the cell proliferation rate of HCE cells treated with or without EGF after scratch injury. **$p < .01$, ***$p < .001$. (f and g) Wound-healing assay indicating that cell migration was enhanced after EGF treatment. (h and i) Flow cytometry detection of apoptosis in cells treated with or without EGF after scratch injury. **$p < .01$, ***$p < .001$. CCK-8, cell counting kit-8; EdU, 5-ethynyl-2′-deoxyuridine; EGF, epidermal growth factor; HCE, human corneal epithelial cell; ROS, reactive oxygen species

3.6 | EGF plays a role in HCE damage repair and mitochondrial autophagy via activation TRPM2

To further determine if EGF indeed plays a role in the activation of TRPM2 pathway, we first demonstrated that the TRPM2 pathway plays a role in HCE damage repair and mitochondrial autophagy. A wound-healing assay indicated that the TRPM2 inhibitor CLT at 20 μM could significantly reduce HCE cell migration of scratch injured HCE cells; while intracellular adenosine diphosphate ribose (cADPR: 10 μM, CAS 119340-53-3) could significantly increase cell migration (*$p < .05$; Figure 6a,b). Furthermore, CLT could inhibit the increase in cell migration induced by EGF (*$p < .05$, **$p < .01$; Figure 6c,d). When

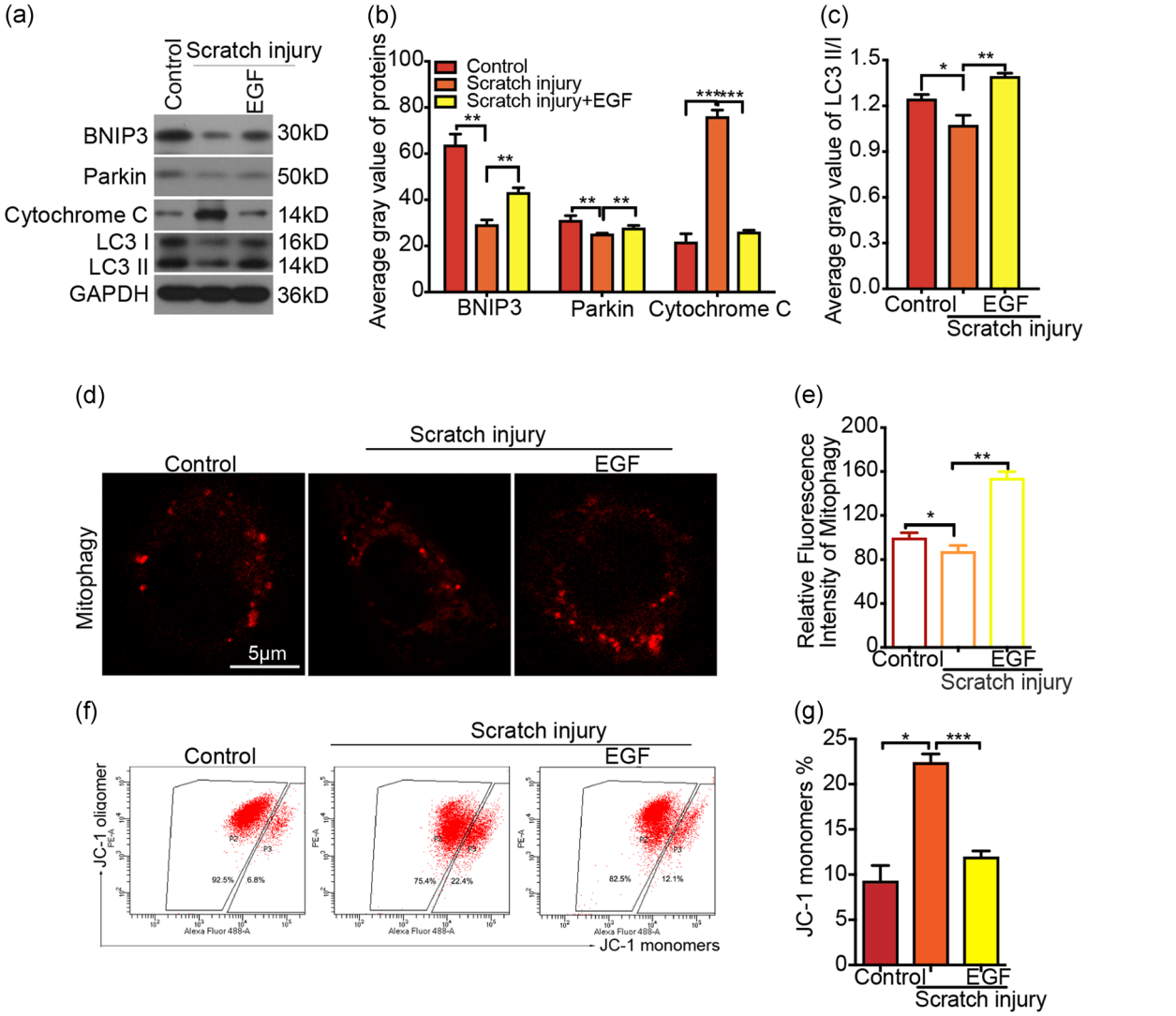

FIGURE 2 EGF could induce mitochondrial autophagy and alleviate mitochondrial damage. (a–c) Western blot determining the expression of BNIP3, Parkin, Cytochrome *c*, and LCII/I following treated with or without EGF in condition of scratch injury. (d and e) Fluorescence was used to detect the intensity of mitochondrial autophagy. *$p < .05$, **$p < .01$. (f and g) JC-1 staining was used to detect the changes of mitochondrial damage. *$p < .05$, ***$p < .001$. BINP3, BCL2 interacting protein 3; EGF, epidermal growth factor; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; LCI; LCII; Parkin, Parkin RBR E3 ubiquitin protein ligase

compared with EGF group, the cell proliferation and cell viability were significantly decreased after CLT combined with EGF treatment in scratch injured HCE cells (*$p < .05$, **$p < .01$, ***$p < .01$; Figure 6e–g). In addition, CLT could significantly inhibit the increase in apoptosis and the autophagy fluorescence induced by EGF in scratch injured HCE cell (*$p < .05$, **$p < .01$, ***$p < .01$; Figure 6h,i). Finally, Western blot analysis was used to determine the autophagy-related protein expression. The results indicated that EGF combined with CLT could downregulate BNIP3 and Parkin protein expression levels and upregulate the levels of Cytochrome C, compared with those in EFG-treated, scratch injured HCE cells (*$p < .05$, **$p < .01$, ***$p < .01$; Figure 6j,k). These finding indicated that EGF plays an important role in HCE damage repair and mitochondrial autophagy via activation of TRPM2.

4 | DISCUSSION

The cornea is a highly specialized organ that must maintain its transparency to function properly. The location of the cornea on the outermost surface of the eye means that it can be damaged by scratch injury, and bacterial and fungal infections. Maintaining a healthy cornea or repairing a damaged cornea is essential for normal vision. Therefore, it is important to explore the mechanism of corneal scratch repair to ensure the integrity of the eye.

EGF exerts an essential biological activity, and is a single polypeptide molecule with three intermolecular disulfide bonds. It is very important in promoting corneal epithelial cell growth, and the corneal epithelial cell is rich in EGF receptors (Imanishi et al., 2000).

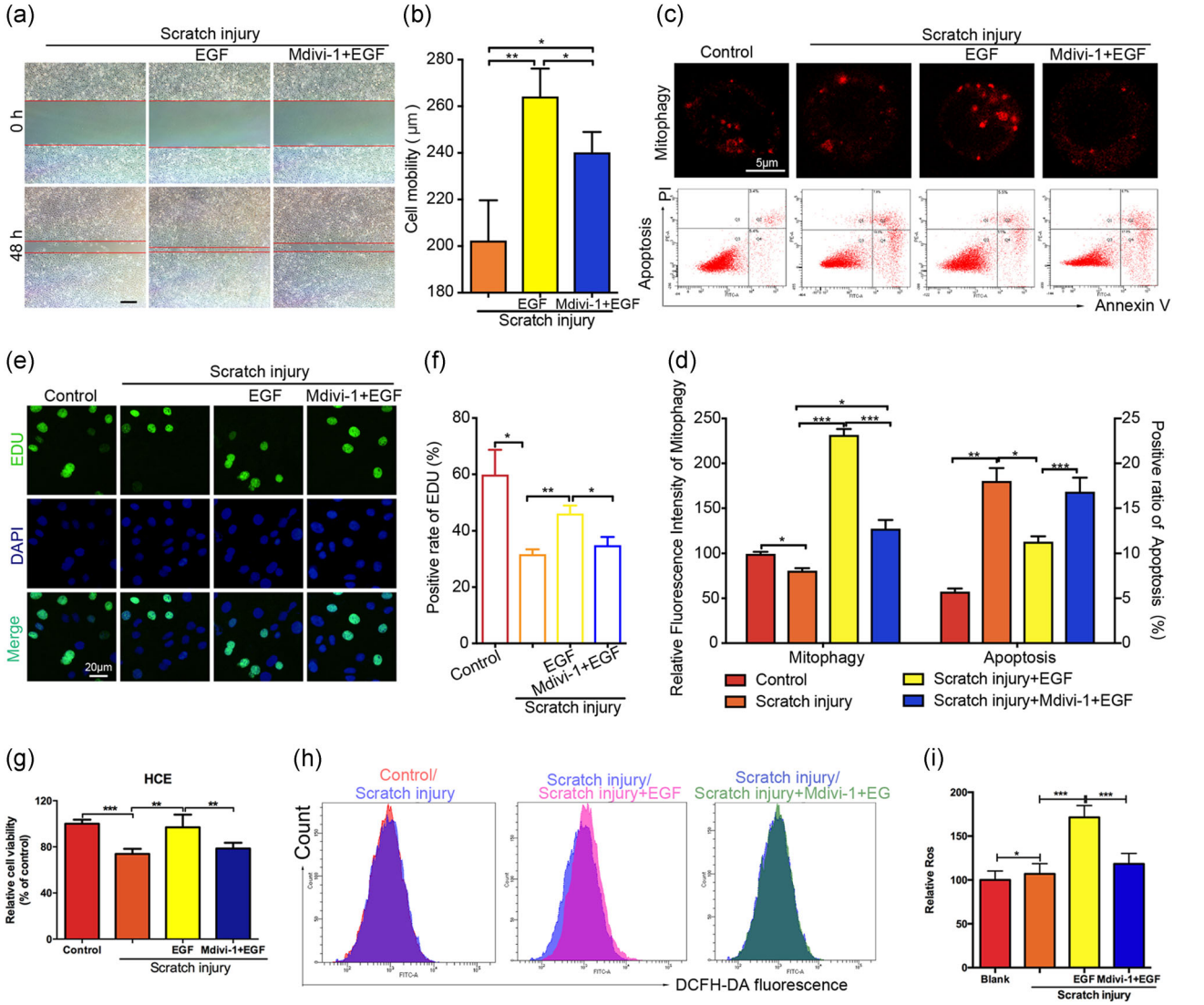

FIGURE 3 EGF could play an important role in inducing mitochondrial autophagy. (a and b) HCE cell layers were subjected to a scratch injury and treated with EGF and EGF combined with MDIVI-1, and the cell migration ability was examined using a wound-healing assay. *$p < .05$, **$p < .01$. (c and d) Fluorescence detection of mitochondrial autophagy and flow cytometry were used to examine HCE cell apoptosis. *$p < .05$, **$p < .01$, ***$p < .001$. (e and f) EdU assay showing that MDIVI-1 combined with EGF could reduce the increase in cell proliferation induced by EGF. *$p < .05$, **$p < .01$. (g) Cell viability was determined using the CCK-8 assay. **$p < .01$, ***$p < .001$. (h and i) Flow cytometry detection of ROS levels after treatment with or without EGF or EFG plus MDIVI-1 under conditions of scratch injury. CCK-8, cell counting kit-8; EdU, 5-ethynyl-2′-deoxyuridine; EGF, epidermal growth factor; HCE, human corneal epithelial cell; MDIVI-1, mitochondrial division inhibitor 1; ROS, reactive oxygen species

Previous studies revealed that EGF could activate ROS and the EGF-induced ROS could promote cell growth, adhesion, and migration (Huo et al., 2009; Huo et al., 2015). High levels of intracellular ROS cause oxidative stress, leading to a variety of pathologies, such as cell death. ROS are the essential mediator for growth factor-stimulated cell proliferation and wound healing in the cornea. As a major source of ROS production, mitochondria are especially prone to ROS damage, which can induce mitochondrial permeability transition by opening nonspecific high conductance permeability transition pores in the mitochondrial inner membrane, leading to cell injury. In the present study, we found that EGF stimulation could increase the level of ROS in scratch injured HCE cells. Moreover, a series of experiments, including CCK-8, EdU, wound healing, and flow cytometry assays, demonstrated that EGF treatment could enhance cell viability, cell proliferation, and migration, and reduce cell apoptosis in scratch injured HCE cells.

Autophagy is an intracellular evolutionarily conserved process, which isolates long-lived proteins from dysfunctional cytoplasmic organelles, and transports them to lysosomes for degradation to maintain cellular homeostasis and avoid cellular damage (Galluzzi, Pietrocola, Levine, & Kroemer, 2014; Marino, Madeo, & Kroemer, 2011). At an early stage of autophagy, the cytoplasmic form of microtubule-associated protein LC3I is coupled with phosphatidylethanolamine to form LC3-II. Thus, LC3-II is considered as a marker for early stage of autophagy (Rezabakhsh

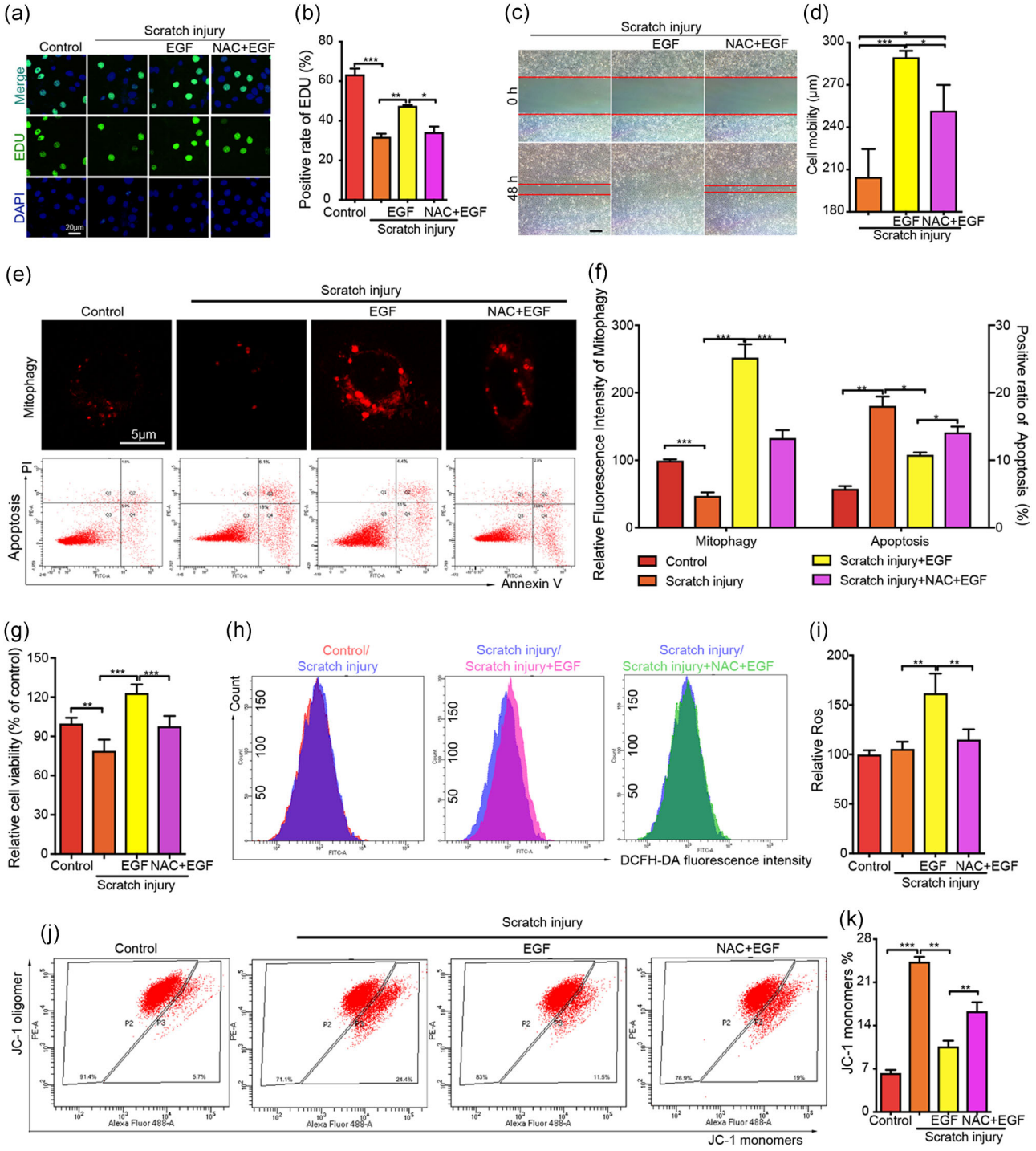

FIGURE 4 The effect of EGF on cell damage and the induction of mitochondrial autophagy disappeared after treatment with the ROS inhibitor NAC. (a and b) EdU assay to detect cell proliferation. *$p < .05$, **$p < .01$. (c and d) Wound-healing assay to detect cell migration of HCE cells. *$p < .05$, **$p < .01$. (e and f) Fluorescence and flow cytometry were used to examine mitochondrial autophagy and HCE cell apoptosis in different groups, respectively. *$p < .05$, **$p < .01$, ***$p < .001$. (g) CCK-8 assay determining cell viability in the different groups. *$p < .05$, ***$p < .001$. (h and i) ROS levels as detected using flow cytometry. **$p < .01$. (j and k) JC-1 staining to detect mitochondrial damage. **$p < .01$, ***$p < .001$. CCK-8, cell counting kit-8; EGF, epidermal growth factor; EdU, 5-ethynyl-2′-deoxyuridine; HCE, human corneal epithelial cell; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species

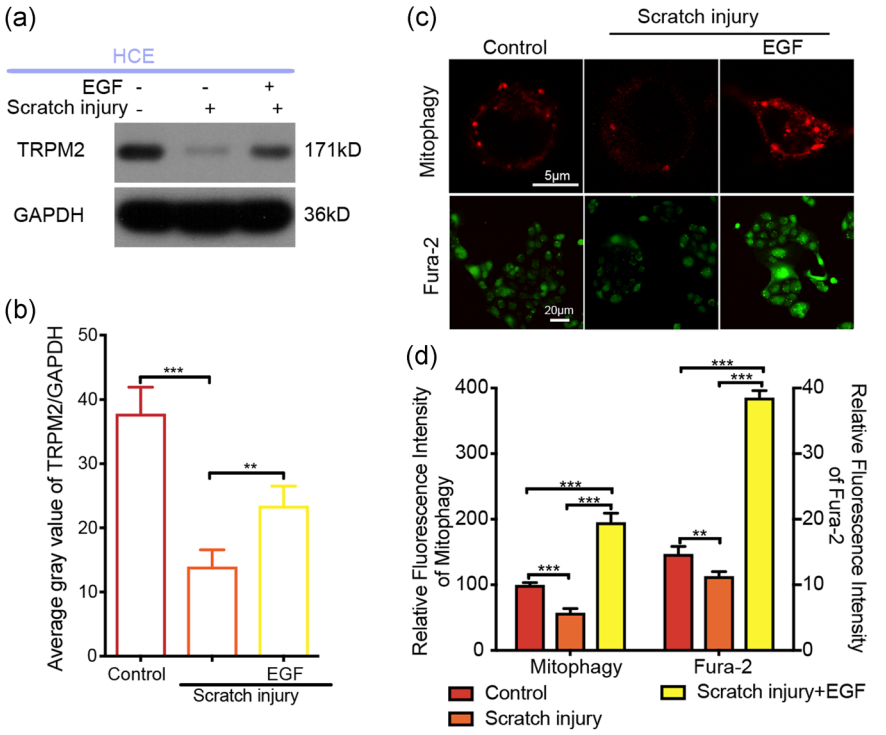

FIGURE 5 The expression of TRPM2 and the concentration of Ca^{2+} were increased after treatment with EGF. (a and b) The TRPM2 level was determined following treatment with or without EGF in HCE cells subjected to scratch wounding or normal HCE cells with no treatment, using Western blot analysis. $^{**}p < .01$, $^{***}p < .001$. (c and d) Fluorescence detection of mitochondrial autophagy and Fura-2 AM. $^{**}p < .01$, $^{***}p < .001$. EGF, epidermal growth factor; HCE, human corneal epithelial cell; Fura-2 AM, Fura-2-acetoxymethyl ester; TRPM2, transient receptor potential cation channel subfamily M member 2

et al., 2017a). The autophagic response could reduce the damage of different cells, but prolonged autophagy can lead to activation of coregulators related to various cell death pathways such as apoptosis (Rezabakhsh et al., 2017b). An important regulator of autophagy is adenosine monophosphate-activated protein kinase. Mitophagy is the specific engulfment of damaged mitochondria by autophagosomes. Under oxidative stress, the oxidative components of the mitochondrial matrix and inner membrane are classified, incorporated into mitochondrial-derived vesicles in a PINK1/Parkin-dependent manner, and then passed to lysosomes (McLelland, Soubannier, Chen, McBride, & Fon, 2014; Soubannier et al., 2012). Mitophagy' s PINK1/Parkin pathway is thought to maintain the mitochondrial quality by eliminating damaged mitochondria, which is regarded as a defense mechanism of mitochondrial function (Kurihara et al., 2012). Autophagy/mitophagy activation contributes to remove the damaged or redundant organelles metabolites and maintain the cell homeostasis(Song et al., 2019). As a major source of ROS production, mitochondria are especially prone to ROS damage, which can induce mitochondrial permeability transition by opening nonspecific high conductance permeability transition pores in the mitochondrial inner membrane, leading to cell injury (Perfeito, Cunha-Oliveira, & Rego, 2013). TRPM2 is a Ca^{2+} permeable cation channel that is primarily activated by cADPR during oxidative stress. TRPM2 is widely expressed in animal tissues and various cancer lines, making it difficult for these cell lines to resist oxidative stress-induced cell death (Hara et al., 2002; Homma et al., 2013; Liu et al., 2013). TRPM2 has been identified to play an essential role in oxidative stress and it has become a potential therapeutic target for oxidative stress-related diseases, such as neurodegenerative diseases, diabetes, and myocardial infarction (Takahashi, Kozai, Kobayashi, Ebert, & Mori, 2011). Ca^{2+} is essential for ROS-mediated autophagy regulation. Numerous studies indeed have already documented that intracellular Ca^{2+} can differentially modulate autophagy upon the context of time, space, Ca^{2+} source, and cell status (Decuypere, Bultynck, & Parys, 2011).Oxidative stress activates a TRPM2-induced Ca^{2+} influx to inhibit autophagy and thus cause cells to become more susceptible to damage, while oxidative stress induced autophagy in the absence of the TRPM2-mediated Ca^{2+} influx (Q. Wang et al., 2016). We hypothesized that the ability of oxidative stress to activate TRPM2-Ca^{2+} signaling might induce mitochondrial autophagy to inhibit cell apoptosis. In the present study, we studied the mechanism of ROS-TRPM2-mediated Ca^{2+} influx in autophagy regulation and demonstrated that oxidative stress triggers Ca^{2+} influx via TRPM2 and induced autophagy to protect HCE cells from injury. EGF treatment could enhance the fluorescence intensity of mitophagy and value of mitochondrial membrane potential. Western blot analysis showed increased levels of LC3-II/LC3I after treatment with EGF in scratch injured HCE cells compared with those in untreated scratch injured cells, implying that this increase in the level of LC3-II/LC3I either occurs through induction of autophagy or blockage of autophagosomal maturation and degradation(Fass, Shvets, Degani, Hirschberg, & Elazar, 2006). Furthermore, it could change the expression of autophagy-related proteins. When we used the autophagy inhibitor MDIVI-1 or the ROS inhibitor NAC combined with EGF to treat scratch injured HCE cells, the protective effect of EGF in HCCLTE cells disappeared, indicating that EGF could have an important effect in inducing mitochondrial autophagy. The present results showed that EGF treatment could increase the decreased in the fluorescence intensities of mitophagy and Fura-2 AM after scratch injury, while the TRPM2 inhibitor CLT combined with EGF, abrogated the protective effect of EGF in scratch injured HCE cells.

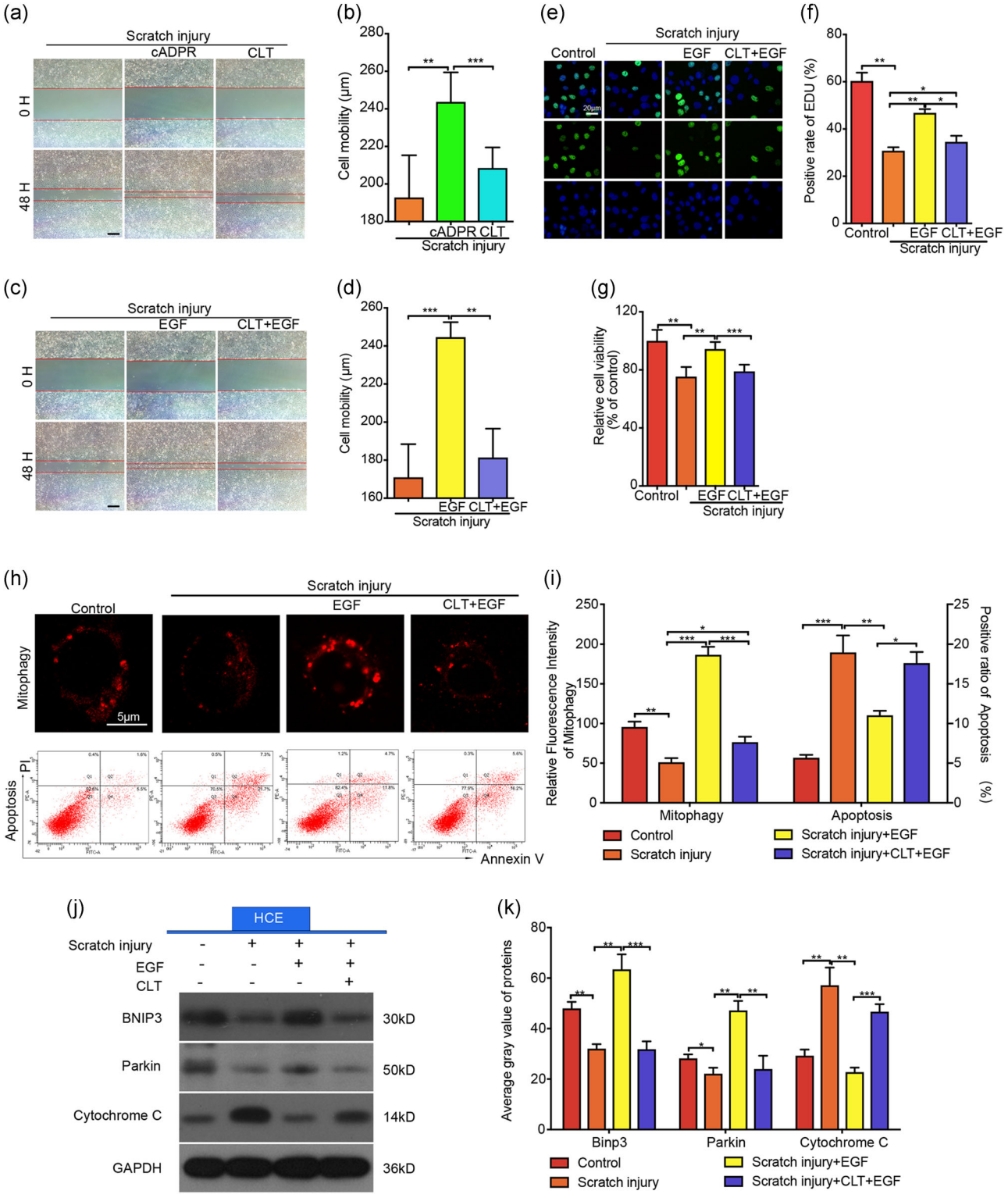

FIGURE 6 EGF plays a role in HCE damage repair and mitochondrial autophagy via activation of TRPM2. (a–d) Wound-healing assay to detect the cell migration ability in HCE cells. $^{**}p < .01$. (e and f) EdU assay to detect cell proliferation after treatment with EGF or EGF combined with CLT in scratch injured HCE cells or controls. $^{**}p < .01$. (g) CCK-8 assay determining cell viability after treatment with EGF or EGF combined with CLT in scratch injured HCE cells or controls. $^{***}p < .001$. (h and i) Fluorescence and flow cytometry were used to examine mitochondrial autophagy and HCE cell apoptosis in the different groups, respectively. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. (j and k) Western blot analysis to examine the expression of BNIP3, Parkin, and Cytochrome *c* protein after treatment with EGF or EGF combined with CLT in scratch injured HCE cells or controls. $^{**}p < .01$, $^{***}p < .001$. BINP3, BCL2 interacting protein 3; CCK-8, cell counting kit-8; CLT, clotrimazole; EdU, 5-ethynyl-2′-deoxyuridine; EGF, epidermal growth factor; HCE, human corneal epithelial cell; Parkin, Parkin RBR E3 ubiquitin protein ligase

In summary, the results of the present study revealed that treatment with EGF could induce mitochondrial autophagy and reduce mitochondrial damage in HCE cells. We provided experimental evidence that EGF activates the TRPM2 pathway in HCE cells by upregulating ROS to cause calcium influx, thereby further inducing mitochondrial autophagy and inhibiting apoptosis, and ultimately protecting HCE cells from damage and promoting repair. This evidence will increase our understanding of the association between scratch injured HCE cells and mitochondrial autophagy mechanisms. EGF-activated ROS plays an important role in HCE damage repair and mitochondrial autophagy via activation of TRPM2.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTION

Y. H. and X. J. conceived the research idea; X. Z., Y. H., Z. J. and Z. Q. performed the experiments; Y. C. and X. L. analyzed the data; Y. H. and W. C. wrote the manuscript. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

Data availability statement We declare that all data supporting the conclusions of the study.

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