# Sleep deprivation induces corneal epithelial progenitor cell over-expansion through disruption of redox homeostasis in the tear film

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## SUMMARY

Sleep deficiency, a common public health problem, causes ocular discomfort and affects ocular surface health. However, the underlying mechanism remains unclear. Herein, we identified that short-term sleep deprivation (SD) resulted in hyperproliferation of corneal epithelial progenitor cells (CEPCs) in mice. The expression levels of p63 and Keratin 14, the biomarkers of CEPCs, were upregulated in the corneal epithelium after short-term SD. In addition, SD led to elevated levels of reactive oxygen species (ROS), and subsequent decrease in antioxidant capacity, in the tear film. Exogenous hydrogen peroxide ($H_2O_2$) could directly stimulate the proliferation of CEPCs *in vivo* and *in vitro*. Topical treatment of antioxidant L-glutathione preserved the over-proliferation of CEPCs and attenuated corneal epithelial defects in SD mice. Moreover, the activation of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway is essential to ROS-stimulated cell proliferation in CEPCs. However, long-term SD ultimately led to early manifestation of limbal stem cell deficiency.

## INTRODUCTION

Sleep is a fundamental biological requirement for all animals. Appropriate quality and quantity of sleep is essential for the maintenance of mental and physical health (Sejnowski and Destexhe, 2000; Wagner et al., 2004). Due to the societal pressures and light pollution, sleep deficiency has become a common public health problem, affecting approximately 10%–20% of individuals worldwide, especially children and young adults (Chattu et al., 2018; Czeisler, 2013; Luyster et al., 2012).

In recent years, sleep-deficiency-induced eye problems have drawn much attention in both the public and medical domains. Short-term consequences of insufficient sleep or delayed sleep cause ocular discomfort, including dryness, pain, pruritus, and hyperemia of the eye (Ayaki et al., 2016; Kawashima et al., 2016). Long-term sleep deprivation (SD) increases risk for ocular disease conditions, especially dry eye disease, affecting 5% to 50% of the population globally (Stapleton et al., 2017). A retrospective study in the United States found that sleep apnea was associated with an increased risk of dry eye (Galor et al., 2011). A large population survey in the Netherlands pointed out that 45% of the dry eye disease participants reported having poor sleep quality (Magno et al., 2021). Similar results have also been concluded in other clinical studies (Kawashima et al., 2016; Yu et al., 2019). A recent

study from our group using an SD mouse model demonstrated that SD could compromise lacrimal gland function and induce dry eye (Li et al., 2018a). Further study found that SD could affect microvilli morphology of corneal epithelial cells through sequential downregulation of PPARα, TRPV6 expression, and Ezrin phosphorylation status in mice (Tang et al., 2018).

The cornea is the clear front surface of the eye. Its overlying tear film (TF) maintains ocular comfort while providing a physical and biochemical barrier to the eye (Maurice, 1957). The corneal epithelial layer is a highly organized structure with adequate stem cells to maintain the homeostasis (Thoft and Friend, 1983). In humans, corneal epithelial stem cells are confined to the basal epithelial layer of the corneal limbus and are referred to as limbal stem cells (LSCs) (Tseng, 1989). LSCs have high capacity for proliferation and express several biomarkers including p63, BCRP1/ABCG2, and Keratin 14 (KRT14) and 19 (Collin et al., 2021; de Paiva et al., 2005; Pellegrini et al., 2001; Schlötzer-Schrehardt and Kruse, 2005). By contrast, the localization of murine corneal epithelial stem cells is more complex and controversial. Corneal epithelial stem cell markers are expressed in the entire basal layer of the corneal epithelium, and slow-cycling cells were detected in both limbal and central murine cornea (Li et al., 2017; Majo et al., 2008). Despite this discordance, it is well accepted that murine corneal basal epithelial cells have
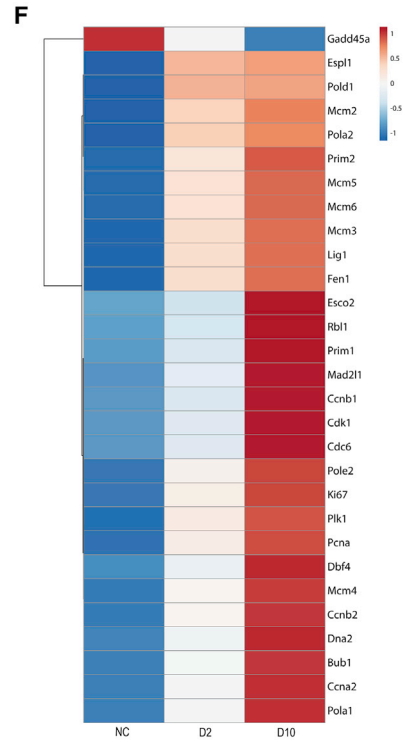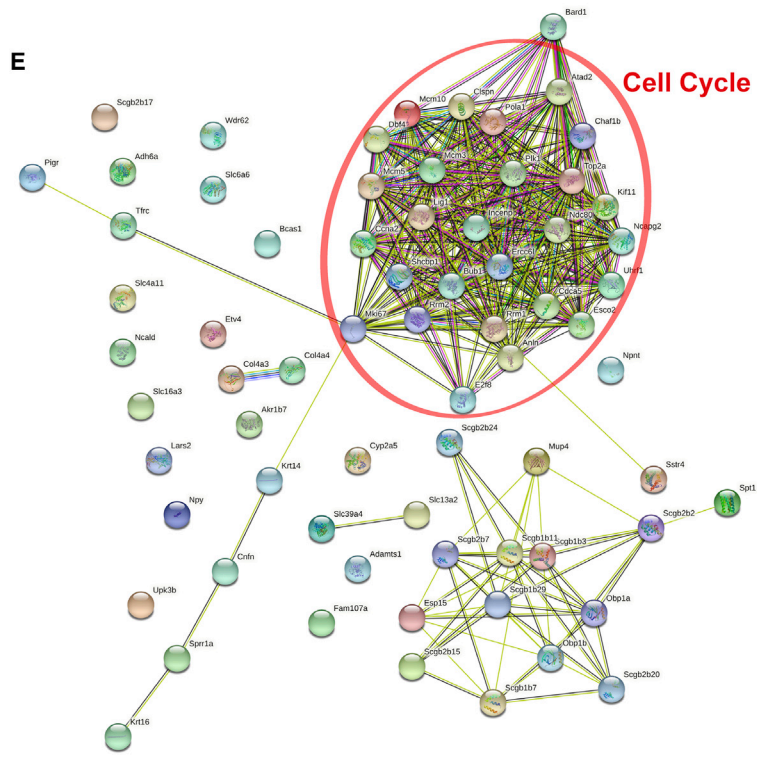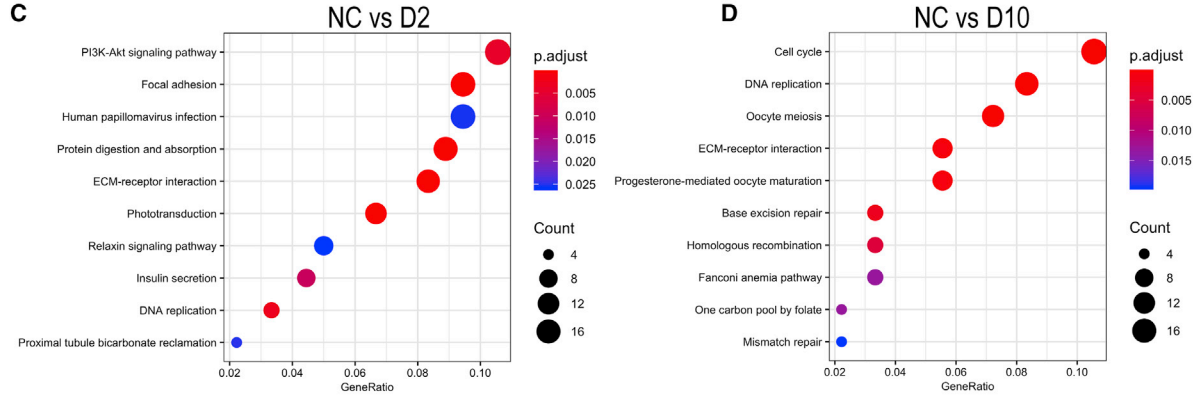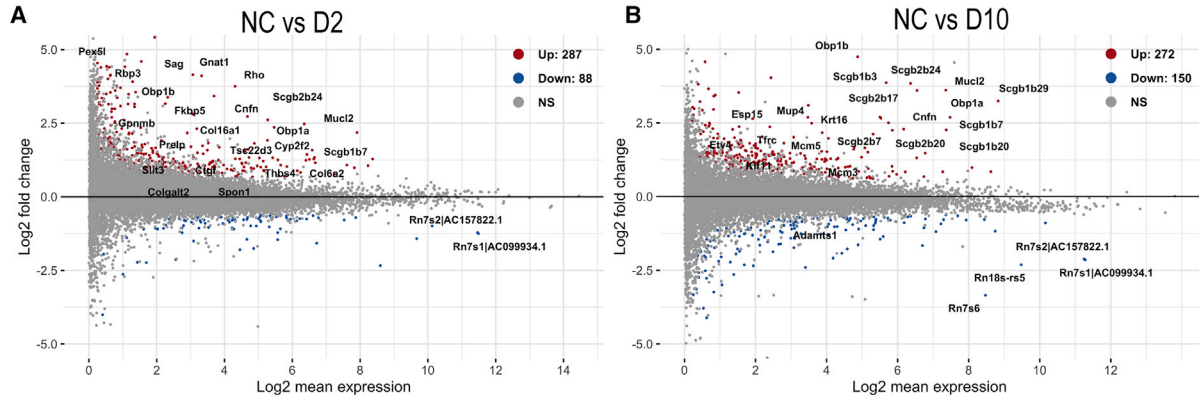
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## RESULTS

### Short-term SD induces hyperproliferation of corneal epithelial cells

To evaluate the effects of SD on corneal epithelial cells, we first analyzed the whole gene expression in corneal epithelium at day 2 and day 10 time points after SD by RNA sequencing (RNA-seq) analysis. The differentially expressed genes (DE-Gs) were identified in the mice corneal epithelium under SD condition. The result showed that a total of 287 genes were significantly upregulated and 88 were downregulated in corneas following 2 days of SD, compared with control cornea (Figure 1A). At the day 10 time point, there were 272 significantly upregulated genes and 150 downregulated genes (Figure 1B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway Gene Ontology enrichment revealed that the most significant and enriched pathway is the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway at day 2 time point. In addition, DNA replication is one of the top 10 enriched pathways (Figure 1C). At the day 10 time point, cell cycle and DNA replication became the most enriched pathways in the gene sets (Figure 1D). To visualize the correlation patterns between DE-Gs, the top 80 DE-Gs at day 10 time point were utilized by using weighted gene co-expression network analysis. Notably, the genes in the most remarkable module are cell-cycle-regulated genes (Figure 1E). The heatmap showed the expression level changes of 29 DE-Gs involved in cell cycle or DNA replication (Figure 1F).

To confirm the RNA-seq results, we then performed RT-qPCR to validate *Ki67*, *Cdk1*, *Esco2*, *Mcm5*, and *Pcna* genes, which are involved in DNA replication or cell proliferation (Bender et al., 2020; Kubben et al., 1994; Pathmanathan and Balleine, 2013; Santamaría et al., 2007; Vetro et al., 2017). The results showed that expression of all the five genes was upregulated in corneal epithelium at the day 5 or day 10 time point after SD, which is consistent with RNA-seq data (Figures 2A–2E).

The upregulation of these genes in sleep-deprived cornea suggests that the proliferation of CEPCs may be promoted under SD condition. To provide direct evidence, we first visu-alized KI67-positive cells by immunofluorescent staining to identify proliferating cells. As predicted, KI67-positive cells were mainly located in the basal layer of corneal epithelia in both the central cornea and limbal area (Figure 2F). However, there was an increase of KI67-positive cells in both areas in SD mice at day 5 and day 10 time points (Figure 2F). Cell counting confirmed a significant increase of KI67-positive cell rate in limbal and central corneal epithelial cells compared with normal control (NC) (Figures 2G and 2H).

We further performed 5-Ethynyl-2'-deoxyuridine (EdU) injection to label the proliferating cells in the DNA synthesis phase (Salic and Mitchison, 2008). The EdU staining showed that the majority of labeled cells resided in the basal layer of the corneal epithelium (Figure 2I). Consistent with the increased rate of KI67-positive cells, the EdU-positive cells in both central and limbal areas were significantly increased after 5 or 10 days of SD (Figures 2J and 2K). Taken together, these data suggest that proliferation of CEPCs was activated during short-term SD.

### Corneal epithelial progenitor cell pool expands after short-term SD

The biomarkers p63 and KRT14 are commonly used to assess the corneal epithelial progenitor cells (CEPCs). Immunofluorescent staining showed that p63 and KRT14 were highly expressed in basal epithelial cells in normal murine corneas. In sleep-deprived mice, however, there were more p63- and KRT14-positive cells in the suprabasal corneal epithelial layer beside the basal layer after 5 or 10 days of SD, compared with age-matched normal mice (Figures 2L and 2M). Analysis of the p63 and KRT14 expression by western blot and qRT-PCR demonstrated that protein expressions (Figure 2N), as well as mRNA expressions (Figures 2O and 2P), were dramatically upregulated in corneal epithelium at day 5 and day 10 of SD, which is also consistent with RNA-seq analysis (Table S1). Altogether, these results suggest that the CEPC pool expanded after short-term SD.

### SD elevates tear ROS levels and decreases tear antioxidant capacity

SD induces oxidative stress in many tissues (Noguti et al., 2013; Villafuerte et al., 2015). To investigate whether SD alters ROS levels in the corneal epithelium and/or its environment tear film, we first assessed the expression of active $H_2O_2$ in the TF and corneal epithelial cells. The ELISA result

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**Figure 1. The transcriptional program in sleep-deprived murine corneal epithelium**

(A and B) M-versus-A plot (MA) plot reveals the differentially expressed genes (DE-Gs) in cornea after 2 days (A) and 10 days (B) of SD, compared with control cornea.

(C and D) KEGG pathway Gene Ontology enrichment analysis of DE-Gs at day 2 (C) and 10 (D) time points, compared with control cornea.

(E) Weighted gene co-expression network analysis shows the top 80 DE-Gs interactions.

(F) Heatmap shows the expressing changes of 27 DE-Gs classified in the module of cell cycle.
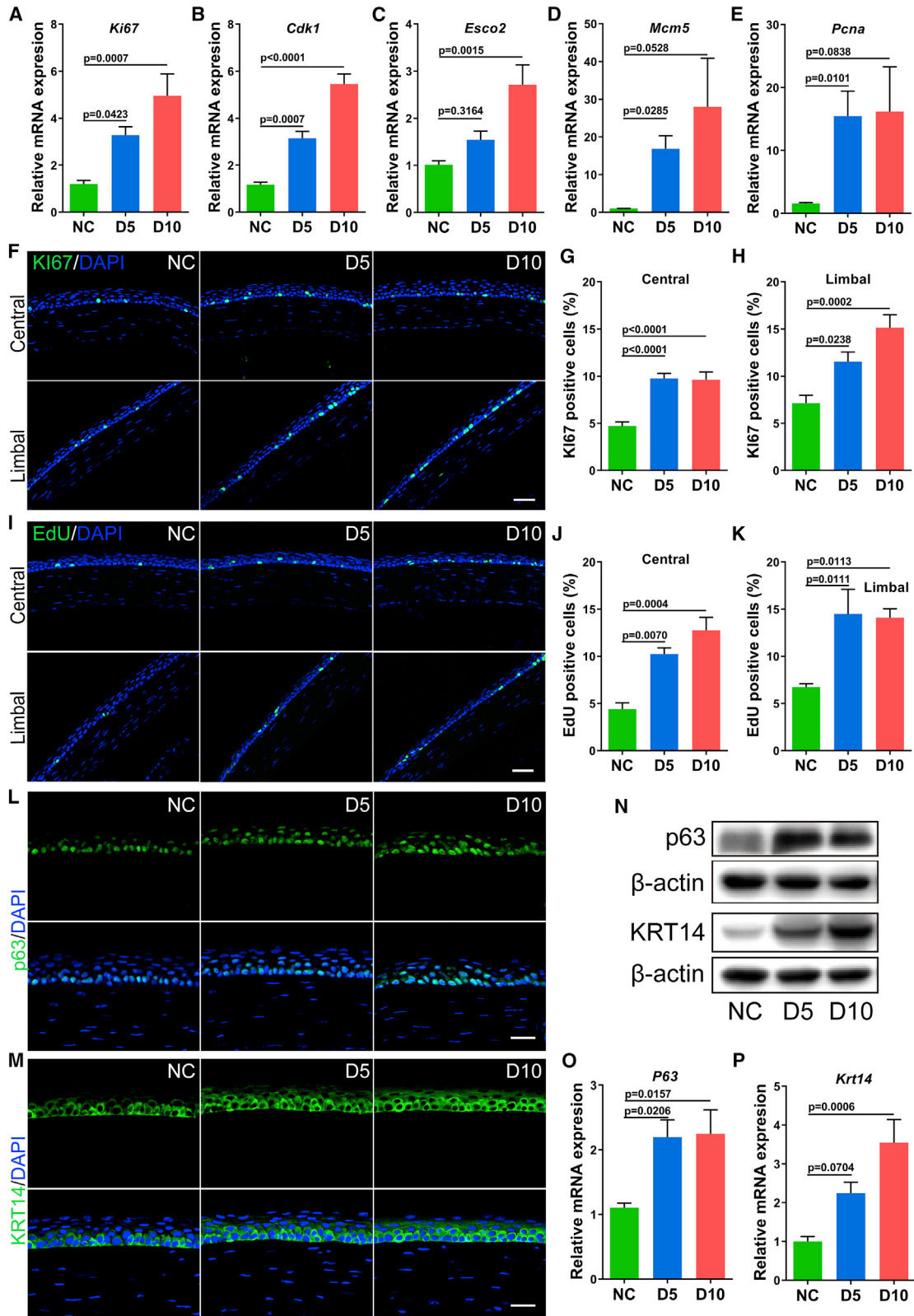
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showed that the active $H_2O_2$ concentration increased almost 3-fold in the TF of mice after 5 days of SD compared with normal mice, and the change was more prominent in mice after 10 days of SD (Figure 3A). By contrast, there was no significant change of active $H_2O_2$ concentration in the corneal epithelial cells (Figure 3B).

Previous studies have shown that the TF has antioxidant capacity (Crouch et al., 1991; Willcox et al., 2017). We then measured the total antioxidant capacity in the TF and corneal epithelial cells using the Ferric Antioxidant Power (FRAP) method. A significant decrease of antioxidant capacity was found in the TF of SD mice at day 5 and day 10, compared with normal mice (Figure 3C). Corneal epithelial cells also showed a significant decrease in antioxidant capacity of SD mice (Figure 3D). Glutathione (GSH) is the most abundant antioxidant in the human body, including the TF (Gukasyan et al., 2007; Townsend et al., 2003). By measuring the GSH concentration, there were no significant changes in the TF of SD mice (Figure 3E). However, the GSH concentration significantly decreased in the corneal epithelial cells after 5 or 10 days of SD (Figure 3F). Glutathione peroxidase (GPX) catalyzes the reduction of peroxide radicals by GSH. To further investigate the potential mechanism of decreased antioxidant capacity in the TF, total GPX activity was quantified in the TF of SD mice and normal mice. GPX activity decreased significantly in the TF after 10 days of SD (Figure 3G). The lacrimal gland serves as the most important exocrine gland to secrete aqueous tear and tear proteins to maintain ocular surface health. We then measured the mRNA expression of GPX families in murine lacrimal glands by qRT-PCR. The expression of *Gpx1* was upregulated after SD for 5 and 10 days (Figure 3H). While *Gpx2* (Figure 3I) and *Gpx3* (Figure 3J) were significantly downregulated after SD for 5 and 10 days, *Gpx4* (Figure 3K) was downregulated after SD for 10 days in lacrimal glands. Overall, ROS level was upregulated while antioxidant capacity was decreased in the TF after short-term SD.

## Exogenous $H_2O_2$ promotes CEPCs proliferation *in vivo*

It was reported that low-dose $H_2O_2$ could stimulate cell proliferation in a wide variety of cell types (Martin and Barrett, 2002; Zhou et al., 2011). To investigate whether the increased tear concentration of peroxide radicals could directly affect the proliferation of CEPCs, we administered various concentrations from 250 μM to 2.5 mM $H_2O_2$ to the ocular surface in normal mice. Using corneal fluorescence staining, we observed a significant epithelial defect after three applications of 2.5 mM $H_2O_2$. However, there was no obvious corneal epithelial defect in 250 and 500 μM $H_2O_2$-treated eyes (Figure 4A). Furthermore, there was no obvious increase of apoptotic cells in the corneal epithelium following three applications of 250 μM or 500 μM $H_2O_2$ based on TUNEL assay, while there was prominent epithelial and stromal cell apoptosis in 2.5 mM $H_2O_2$-treated corneas (Figure 4B).

To evaluate the effect of peroxide radicals on corneal epithelial cell proliferation, KI67 immunostaining was performed. We found obvious increase of KI67-positive cells in the central corneal epithelium after 500 μM $H_2O_2$ application (Figure 4C), which was confirmed by KI67-positive cell counting (Figure 4E). qRT-PCR results also showed that mRNA expression of *Ki67* was significantly upregulated after treatment of $H_2O_2$ at a concentration of 500 μM (Figure 4F). Additionally, EdU-positive cells (Figure 4D) and the positive rate (Figure 4G) also significantly increased after 250 and 500 μM $H_2O_2$ treatment. These results indicate that a relatively low concentration of $H_2O_2$ promoted expansion of CEPCs.

## Topical administration of L-glutathione attenuates hyperproliferation of CEPCs after SD

Given the decreased concentration of GSH in the corneal epithelium of SD mice (Figure 3F), we next asked whether topical treatment of GSH could reverse the hyperproliferation of CEPCs in SD mice. Following topical administration

**Figure 2. SD stimulates CEPC over-expansion**

(A–E) Expression of *Ki67* (A), *Cdk1* (B), *Esco2* (C), *Mcm5* (D), and *Pcna* (E) mRNA in the corneal epithelium after 5 or 10 days of SD, compared with NC mice.

(F) Representative images of KI67 immunofluorescent staining in both central and limbal areas of cornea at days 5 and 10 of SD, relative to NC mice. The scale bar represents 50 μm.

(G and H) Quantification of KI67-positive cells rate in central (G) and limbal (H) corneal epithelium.

(I) Representative images of EdU-positive cells in both central and limbal areas of cornea at days 5 and 10 of SD, relative to NC mice. The scale bar represents 50 μm.

(J and K) Quantification of EdU-positive cells rate in both central (J) and limbal (K) corneal epithelium.

(L and M) Representative images of p63 (L) and KRT14 (M) immunofluorescent staining in central cornea at days 5 and 10 of SD, relative to NC mice. The scale bar represents 30 μm.

(N) p63 and KRT14 were evaluated by western blot in the corneal epithelium of sleep-deprived mice, relative to NC mice.

(O and P) Expression of *P63* (O) and *Krt14* (P) mRNA in the corneal epithelium after 5 or 10 days of SD, compared with NC mice. Data are representative of three independent experiments (mean ± SEM; n = 5–6 mice/group; minimum three images were analyzed from each sample).
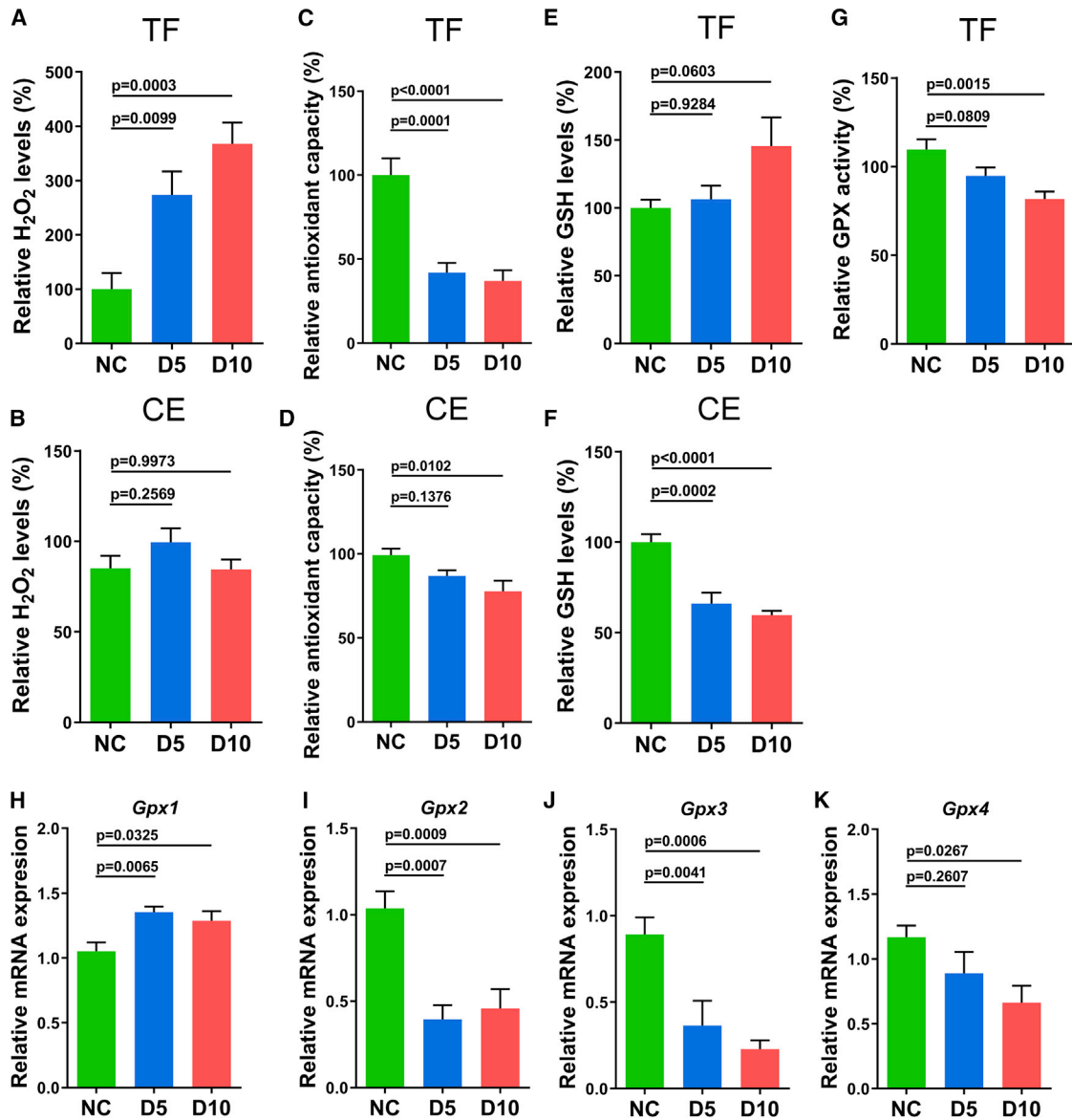
**Figure 3. ROS changes in the ocular surface after SD**

(A and B) $H_2O_2$ levels in the TF (A) and CE (B) from mice at days 5 and 10 of SD, relative to NC mice.

(C and D) Total antioxidant capacity in the TF (C) and CE (D) at days 5 and 10 of SD, relative to NC mice.

(E and F) GSH levels in the TF (E) and CE (F) from mice after 5 days or 10 days of SD, relative to NC mice.

(G) GPX activity in the TF at days 5 and 10 of SD compared with NC mice.

(H–K) Expression of *Gpx1* (H), *Gpx2* (I), *Gpx3* (J), and *Gpx4* (K) mRNA in the lacrimal gland at days 5 and 10 of SD, relative to NC mice. Data are representative of two independent experiments (mean ± SEM; n = 6–8 mice/group). CE, corneal epithelium; TF, tear film.

of 2% reduced glutathione (L-glutathione) to the ocular surface for 2 or 5 days in SD mice, corneal fluorescein staining indicated that SD-induced corneal epithelial defects were largely resolved (Figure 5A). The fluorescein score significantly decreased in 5-day L-glutathione-treated eyes, compared with phosphate-buffered saline (PBS)-treated control eyes (Figure 5B). To evaluate the efficacy

of topical L-glutathione in reducing CEPC hyperproliferation, we next measured the *Ki67*, *Cdk1*, *Esco2*, *Mcm5*, and *Pcna* gene expressions in L-glutathione- or PBS-treated cornea. qRT-PCR results revealed a significant decrease of *Cdk1*, *Esco2*, and *Pcna* gene expressions in the 5-day L-glutathione-treated group (Figures 5D, 5E, and 5G). The relative expression levels of *Ki67* and *Mcm5* genes also
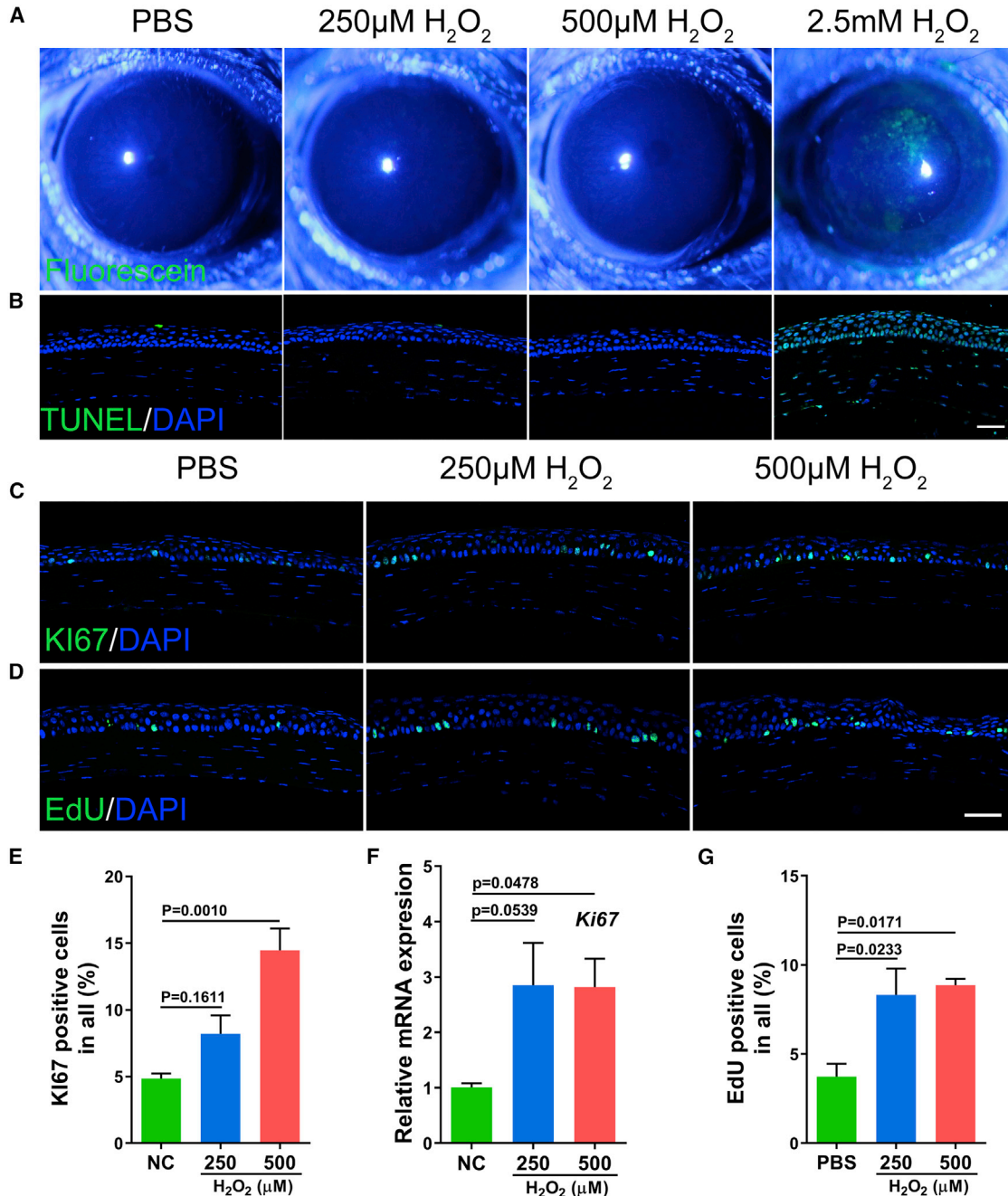
**Figure 4. Exogenous $H_2O_2$ promotes CEPC proliferation *in vivo***

(A) Representative images of corneal fluorescein staining are shown in 250 μM, 500 μM, and 2.5 mM $H_2O_2$-treated eyes, relative to PBS-treated eye.

(B) Representative images of TUNEL staining in central cornea treated with PBS, 250 μM, 500 μM, or 2.5 mM $H_2O_2$. The scale bar represents 50 μm.

(C and D) Representative images of KI67 (C) and EdU (D) fluorescent staining in the central cornea. The scale bar represents 50 μm.

(E–G) Quantification of KI67-positive (E) and EdU-positive (G) cell rate in central corneal epithelium after 250 and 500 μM $H_2O_2$ treatment.

(F) Expression of *Ki67* mRNA in 250 and 500 μM $H_2O_2$-treated cornea, compared with control cornea. Data are representative of two independent experiments (mean ± SEM; n = 4 mice/group; minimum three images were analyzed from each sample).
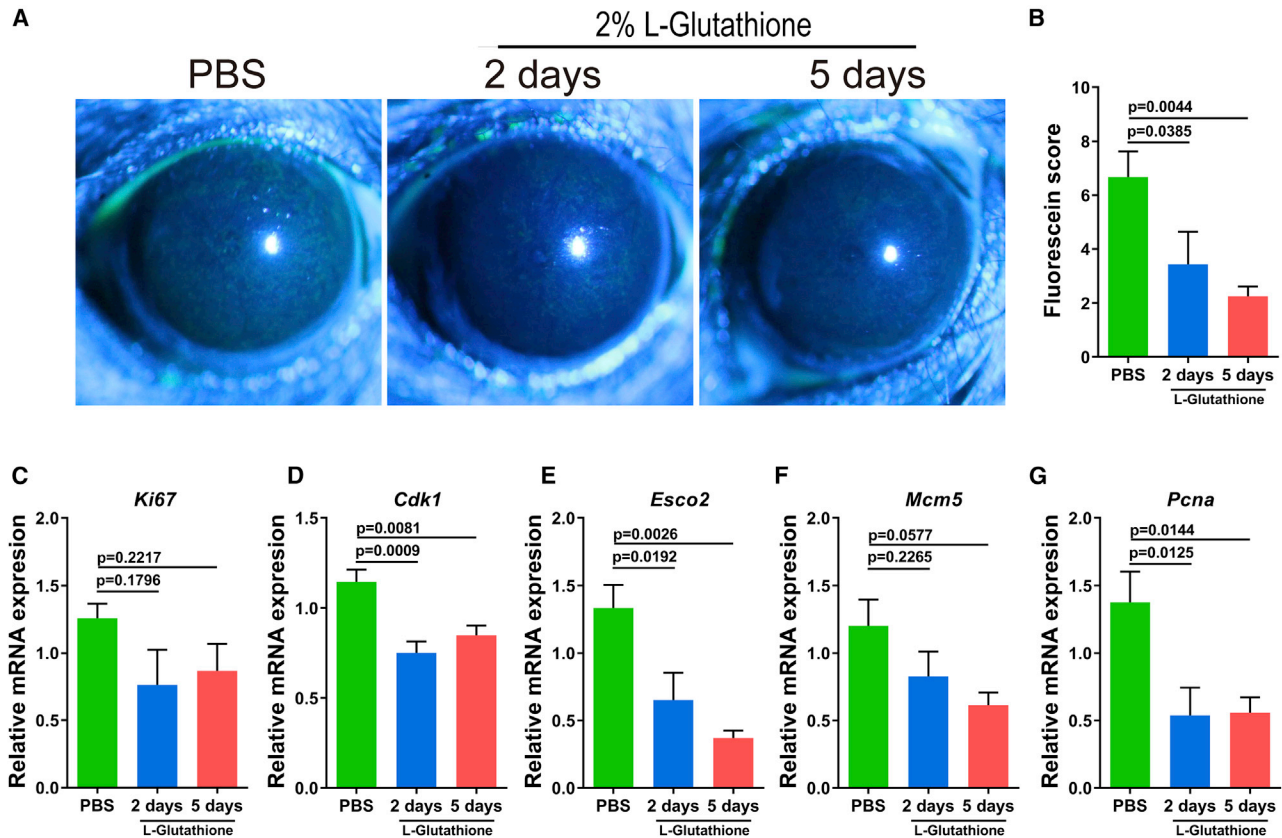
**Figure 5. Topical L-glutathione treatment reduces corneal epithelial defect and attenuates the over-proliferation of CEPCs in SD mice**

(A) Representative images of corneal fluorescein staining in 2- and 5-day 2% L-glutathione eye drop-treated eyes, compared with PBS-treated control eye.

(B) Quantification of corneal fluorescein staining in 2- and 5-day L-glutathione-treated eyes, relative to control eye.

(C–G) Expression of *Ki67* (C), *Cdk1* (D), *Esco2* (E), *Mcm5* (F), and *Pcna* (G) mRNA in the corneal epithelium after 2 and 5 days of L-glutathione treatment, relative to NC mice. Data are representative of two independent experiments (mean ± SEM; n = 5–8 mice/group).

showed a tendency to decrease (Figures 5C and 5F). In sum, these results suggest that L-glutathione eye drops may attenuate hyperproliferation of CEPCs in SD mice.

**Exogenous hydrogen peroxide activates human and mouse CEPCs *ex vivo***

To further validate the effect of peroxide radicals on CEPCs, we applied a series concentration of hydrogen peroxide ($H_2O_2$) (10, 25, 50, 100, and 250 μM) to TKE2 cells, a commonly used cell line for mouse corneal epithelial progenitor/stem cells. After 24 h, the total number of cells was evaluated using the CCK8 assay. Our results indicate that the number of TKE2 cells increased in both 10 and 25 μM $H_2O_2$-treated groups, compared with the untreated control group (Figure 6A). Similarly, a series of lower concentration of $H_2O_2$ (0.625, 1.25, 2.5, 5, 10 μM) were applied to the human primary limbal epithelial cells (hLECs). A

similar effect of $H_2O_2$ stimulating cell proliferation was observed in 2.5 μM concentration groups (Figure 6B).

**PI3K/AKT signaling pathway is indispensable to ROS-mediated hyperproliferation of CEPCs**

The PI3K/AKT signaling pathway plays a well-established role in promoting cell-cycle progression (Chang et al., 2003). Pathway enrichment analysis of RNA-seq data indicates that PI3K/AKT signaling pathway was activated in corneal epithelium after SD (Figure 1C). We proposed that ROS-mediated CEPC proliferation may require activation of the PI3K/AKT signaling pathway. To verify the activation of PI3K/AKT signaling pathway, we detected the expression of phosphorylated AKT (pAKT) in corneal epithelium after SD. The western blot result showed pAKT was upregulated in both 5- and 10-day sleep-deprived corneal epithelium (Figure 6C). To determine whether
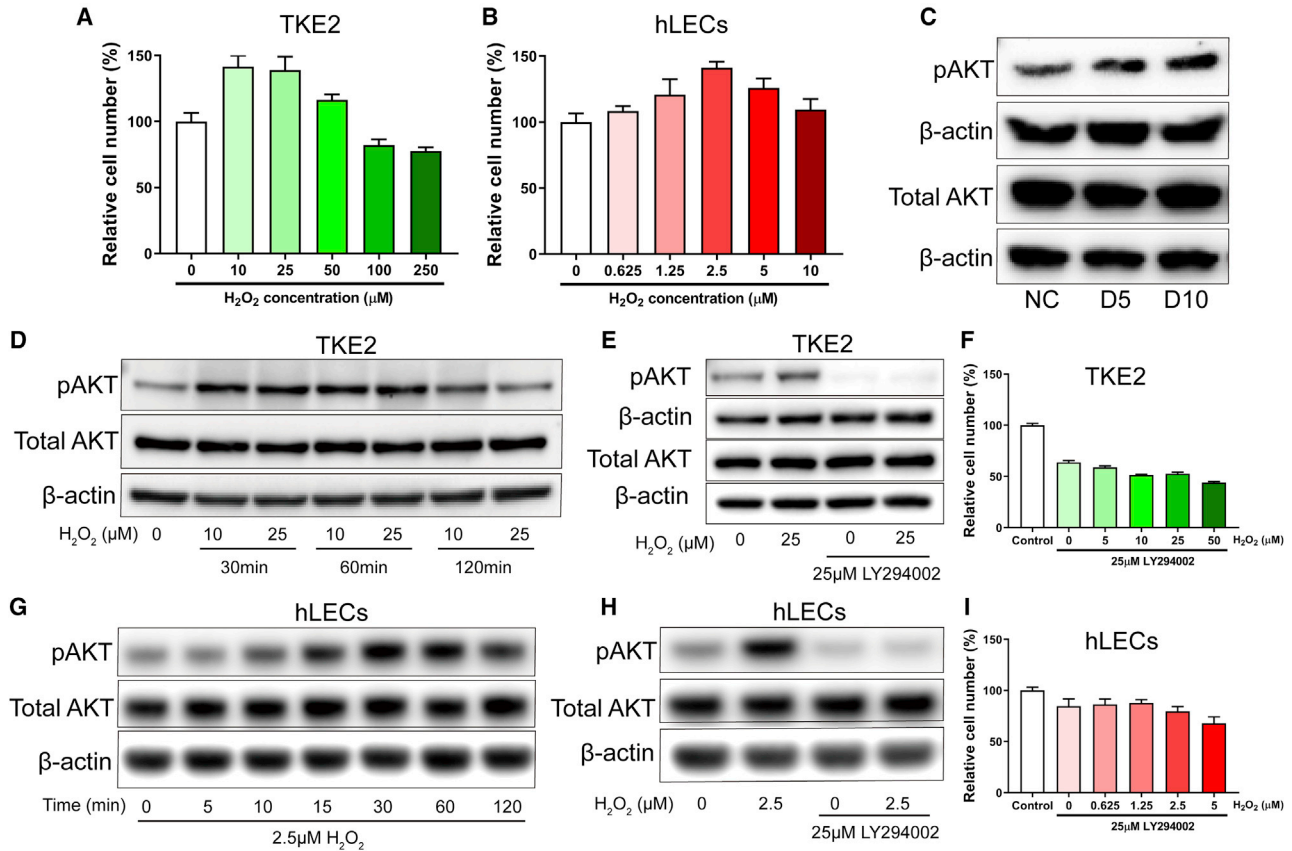
**Figure 6. PI3K/AKT signaling pathway activation is essential for ROS-stimulated cell proliferation in CEPCs**

(A) CCK8 detection in TKE2 cells treated with 10, 25, 50, 100, and 250 μM $H_2O_2$ for 24 h, relative to untreated control cells.

(B) CCK8 detection in hLECs treated with 0.625, 1.25, 2.5, 5, and 10 μM $H_2O_2$ for 24 h, relative to untreated control cells.

(C) Total AKT and pAKT were evaluated by western blot in cornea after SD for 5 and 10 days, relative to NC mice.

(D) Total AKT and pAKT were evaluated by western blot in TKE2 cells treated with 10 or 25 μM $H_2O_2$ for 30, 60 and 120 min.

(E) Total AKT and pAKT were evaluated by western blot in TKE2 cells treated with or without 25 μM $H_2O_2$ and/or 25 μM LY294002.

(F) CCK8 detection in TKE2 cells treated with or without $H_2O_2$ and/or LY294002.

(G) Total AKT and pAKT were evaluated by western blot in hLECs treated with 2.5 μM $H_2O_2$ for 5, 10, 15, 30, 60 and 120 min.

(H) Total AKT and pAKT were evaluated by western blot in hLECs treated with or without 2.5 μM $H_2O_2$ and/or 25 μM LY294002.

(I) CCK8 detection in hLECs treated with or without $H_2O_2$ and/or LY294002. Data of CCK8 assay and western blotting are representative of three independent experiments (mean ± SEM; A and F, n = 6 technical replicates/group; B and I, n = 8–16 technical replicates/group).

exogenous ROS could directly activate the PI3K/AKT signaling pathway in corneal epithelial cells, we then treated TKE2 cells with 10 and 25 μM $H_2O_2$ for 30, 60, and 120 min and detected the total AKT and pAKT expressions by western blot. The results show that pAKT expression was upregulated after treatment with both 10 and 25 μM $H_2O_2$ at 30 and 60 min, and reduced at 120 min (Figure 6D). Total AKT protein expression overall did not change significantly following $H_2O_2$ treatment (Figure 6D). Similarly, pAKT expression in hLECs increased from 10 min to 120 min after 2.5 μM $H_2O_2$ treatment (Figure 6G).

LY294002, a specific inhibitor of PI3Ks, is commonly used to inhibit the PI3K/AKT signaling pathway (Gharbi et al., 2007). To assess the role of PI3K/AKT signaling

pathway in ROS-mediated CEPC proliferation, we treated TKE2 cells and hLECs with LY294002 under normal conditions or $H_2O_2$ stimulation. Our data demonstrated that LY294002 strongly inhibited AKT phosphorylation induced by $H_2O_2$ stimulation in TKE2 cells (Figure 6E) and hLECs (Figure 6H). LY294002 also downregulated the pAKT level in unstimulated cells under normal culture condition (Figures 6E and 6H). To detect LY294002 efficacy on $H_2O_2$-stimulated TKE2 cells and hLECs proliferation, the total number of cells was evaluated after exposure to different concentrations of $H_2O_2$ with or without LY294002. The results showed that the effect of $H_2O_2$ on proliferation of TKE2 cells and hLECs was inhibited under the treatment of 25 μM LY294002 (Figures 6F and 6I). Both
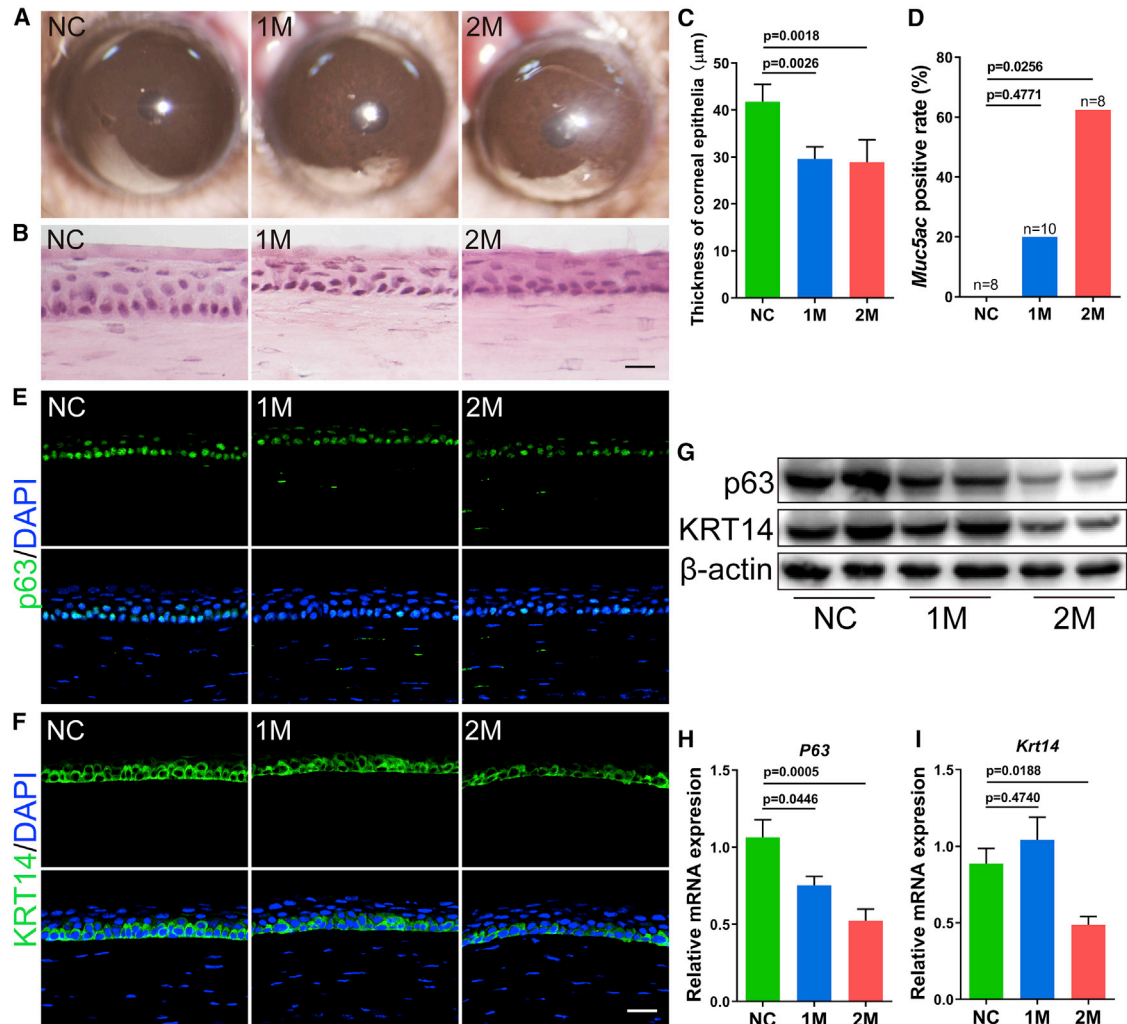
**Figure 7. Long-term SD induces LSCD phenotypes**

(A) Slit-lamp microscopy images of cornea after 1 and 2 months of SD, relative to NC mice. (B) H&E staining images of corneal epithelial layer after SD for 1 and 2 months, relative to NC mice. The scale bar represents 30 μm.

(C) Quantification of corneal epithelial thickness after 1 and 2 months of SD, relative to NC mice.

(D) qRT-PCR analysis showing an increased rate of *Muc5ac*-gene-positive expression in the cornea after 1 and 2 months of SD.

(E and F) Representative immunofluorescent staining images of p63 (E) and KRT14 (F) after SD for 1 and 2 months, relative to NC mice. The scale bar represents 30 μm.

(G) p63 and KRT14 were evaluated by western blot in the corneal epithelium after SD for 1 and 2 months, relative to NC mice.

(H and I) Expression of *p63* mRNA (H) and *Krt14* mRNA (I) in the corneal epithelium after 1 and 2 months of SD, relative to NC mice. Data are representative of three independent experiments. (mean ± SEM; n = 5–10 mice/group).

TKE2 cell and hLECs proliferation were inhibited by LY294002 treatment only (Figures 6F and 6I). Overall, these results indicate that ROS-mediated CEPC proliferation depends on the activation of PI3K/AKT signaling pathway.

## Long-term SD results in corneal epithelial stem cell deficiency

To further determine whether CEPCs could maintain the stemness while keeping expansion after long-term SD, mice were subjected to 1 and 2 months of SD. Slit-lamp microscopy showed that, after 2 months of SD, the cornea was less transparent, and the ocular surface became rough and dry, compared with the healthy control (Figure 7A). Hematoxylin and eosin (H&E) staining revealed that the arrangement of corneal basal epithelial cells was irregular after 2 months of SD (Figure 7B). Remarkably, the thickness of the corneal epithelium was significantly decreased after 1 and 2 months of SD (Figure 7C), which is a diagnostic

measure of limbal stem cell deficiency (LSCD) disease in clinic (Chan et al., 2015). Corneal conjunctivalization is another reliable sign of LSCD (Puangsricharern and Tseng, 1995). The mucin protein MUC5AC is specifically expressed in conjunctival goblet cells but not in corneal epithelial cells. qRT-PCR revealed that two out of 10 corneas were *Muc5ac* positive after 1 month of SD, while five out of eight corneas were positive after 2 months of SD. By contrast, all the healthy control corneas did not express the *Muc5ac* gene (Figure 7D). These results suggest that corneal conjunctivalization occurred after long-term SD.

Next, we assessed the expression of stem cell markers p63 and KRT14 in the corneal epithelium after long-term SD. Immunofluorescent staining results showed that expression of p63 and KRT14 in some of the basal corneal epithelial cells decreased after 2 months of SD (Figures 7E and 7F). Western blot confirmed that both p63 and KRT14 expression decreased after 2 months of SD, compared with the NC corneas (Figure 7G). qRT-PCR data also showed a significant decrease of *p63* and *Krt14* gene expression after 2 months of SD (Figures 7H and 7I). Taken together, these results strongly indicate that long-term SD reduced the stemness in CEPCs.

## DISCUSSION

ROS are generated as a natural byproduct from normal cellular metabolism of oxygen, often following exposure to infectious agents, xenobiotics, inflammation, or other cellular stressors (Pizzino et al., 2017; Redza-Dutordoir and Averill-Bates, 2016; Sies and Jones, 2020). High-quality sleep plays an irrefutable role in maintaining health, in part by defending against oxidative stress (Gopalakrishnan et al., 2004). Conversely, SD has been associated with increases of oxidative stress in numerous tissues and plasma (Gopalakrishnan et al., 2004; Villafuerte et al., 2015). The cornea is exposed to a highly oxidative environment due to its intense exposure to light and high oxygen tension (Álvarez-Barrios et al., 2021). However, the aqueous layer of the TF, secreted by the lacrimal gland, contains various antioxidants, including ascorbic acid, lactoferrin, uric acid, and cysteine, which work in concert to protect the ocular surface against free radical injury (Álvarez-Barrios et al., 2021). Additionally, lacrimal gland-secreted proteins, such as antioxidant enzymes including superoxide dismutase and GPX, make a contribution as antioxidants (Sedlak et al., 2021). This machinery helps maintain the tear redox homeostasis. Our work, for the first time, demonstrates that SD disrupts the redox homeostasis in the TF by increasing the ROS levels and decreasing antioxidant capacity. There are several mechanisms possibly related to the decompensation of the homeostasis. First, the lacrimal

gland may take responsibility for raised ROS level in the TF. Our previous study showed lipid peroxidation and accumulation in mouse lacrimal gland under SD condition, which indicates the increase of ROS levels in the lacrimal gland (Li et al., 2018a). Second, a decrease in the TF antioxidant capacity may result in an increase of tear ROS levels. A decreased expression of *Gpx2*, *Gpx3*, and *Gpx4* genes in lacrimal gland, corresponding with the reduction of GPX activity in the TF, may contribute to decreased total antioxidant capacity in the TF. Interestingly, we did not observe increased ROS levels in corneal epithelial cells after SD. This may be due to more effective antioxidants such as catalase existing in the cytoplasm of corneal epithelial cells, which is not detectable in human TF (Crouch et al., 1991).

Excessive ROS can directly damage cellular proteins, lipids, and DNA, which may further induce cell death (Dickinson and Chang, 2011). An increased level of ROS and oxidative stress have been identified in the tears of dry eye patients (Augustin et al., 1995; Seen and Tong, 2018). Our previous study also identified the dry eye-like phenotypes in SD mice (Li et al., 2018a). However, the causative relationship between dry eye and oxidative stress in the TF is still unclear. In our SD model, we showed that higher ROS level in the TF might be responsible for some pathological changes in the ocular surface under SD-induced dry eye condition. This is supported by the evidence that the corneal epithelial defect was largely reduced after topical application of 2% L-glutathione eye drops in SD mice. On the other hand, ROS or related reactive species act as second messengers in a variety of cellular processes, including cell signaling, homeostasis, and conferment of tolerance to various environmental stresses (Ray et al., 2012). The importance of ROS and redox homeostasis has been recognized in various different stem cell types, particularly those in hematopoietic, neural, and muscle tissues (Chaudhari et al., 2014; Ito et al., 2006; Kobayashi and Suda, 2012). It has become increasingly clear that redox status influences stem cell maintenance, in part through the regulation of the cell cycle. For instance, high endogenous ROS level was observed in neural stem cells, which helps regulate self-renewal and neurogenesis (Le Belle et al., 2011). Oxidative stress was also recognized to be necessary to trigger intestinal stem cell proliferation in *Drosophila* (Xu et al., 2017). However, to the best of our knowledge, no study has investigated the response of adult stem cells to environmental oxidative stress. In this study, we provide both *in vivo* and *in vitro* evidence that environmental ROS alters the biological activity of adult corneal epithelial stem cells. Upregulated ROS in the TF under SD condition activated self-renewal of mice corneal epithelial stem cells, which was approved by markers such as KI67, p63, KRT14, and EdU labeling. Low concentration of ROS could also

activate CEPCs during *in vitro* culture. We further revealed that PI3K/AKT signaling pathway was involved in this CEPC activation process. In homeostatic corneal tissue, a balance must exist between corneal epithelial stem cell proliferation and the desquamation of corneal superficial epithelial cells. Our previous study demonstrated that apoptotic cells increased in the superficial layer of the cornea after 10 days of SD due to dry eye symptoms (Li et al., 2018a). Following short-term SD, CEPCs were stimulated to over-proliferate by environmental ROS in the TF. This response of progenitor cells may serve to replenish the superficial epithelial layer following accelerating cell death. However, this capacity for replenishment may not be sustained in the long term. Indeed, chronic SD for 1 or 2 months, ultimately, led to the depletion of corneal epithelial stem cells and was associated with early manifestation of LSCD. Taken together, these findings may also provide new insight into the pathophysiology of severe dry eye diseases such as Sjögren's syndrome and LSCD.

In conclusion, we have demonstrated that SD increased tear ROS levels but decreased the antioxidant capacity of the TF, which resulted in the hyperproliferation of the CEPCs through the activation of the PI3K/AKT signaling pathway. Moreover, topical L-glutathione treatment may be a therapeutic strategy to maintain ocular surface health for those suffering from insufficient sleep. Further investigation studies are needed to investigate whether administration of L-glutathione eye drops or inhibition of the PI3K/AKT signaling pathway improve the maintenance of CEPCs stemness and whether LSCD syndrome can be reversed after the reintroduction of sufficient sleep.

## EXPERIMENTAL PROCEDURES

### Animals
All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the experimental protocol was approved by the Experimental Animal Ethical Committee of Xiamen University. Details for mice used in the study can be found in supplemental experimental procedures.

### SD model establishment
The "stick over water" method is described in our previous studies (Li et al., 2018a; Tang et al., 2018). Briefly, mice in the SD group were placed on a stick configuration for 20 h per day (5 pm to 1 pm next day) and returned to their home cages during the resting phase. Before the experiment, each mouse was adapted to the SD procedure for 1 h on three consecutive days. Two mice were kept in each cage. The control group mice were housed in standard home cages.

### RNA-seq
Corneal epithelial tissues were collected from four individual mice and mixed for one sample. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) and assessed using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA) and Qubit Fluorometer (Invitrogen, Carlsbad, CA). The RNA quality was evaluated as follows: RNA integrity number >7.0 and 28S:18S ratio >1.8. Libraries for sequencing were constructed with the NEB Next Ultra RNA Library Prep Kit (NEB) for Illumina. The library cDNA was subjected to paired-end sequencing with a pair end 125-base-pair reading length on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA). All statistical analyses were performed using programming language R version 3.6.1. MA plot visualization was achieved by R package ggpubr 0.4.0 (https://CRAN.R-project.org/package=ggpubr). KEGG pathway analysis was performed with R package clusterProfiler 3.14.3 (Yu et al., 2012). Weighted gene co-expression network analysis was conducted by the STRING database (https://string-db.org). The list of DE-Gs is shown in Table S1.

### $H_2O_2$, total antioxidant capacity, GSH and GPX measurements
The SD or control mice were anesthetized with ketamine/xylazine. To harvest tears, 2 μL of PBS was placed on the ocular surface of each eye. Following 10 s of mixture, 2 μL of irrigated tears were harvested by pipette and immediately diluted to 98 μL with PBS. For murine corneal epithelium sample collection, the whole corneal epithelial layer was removed by using a corneal rust ring remover (Algerbrush II, USA) and was immediately transferred into 100 μL of cold PBS. After homogenizing the tissue, the supernatant was collected for further detection. $H_2O_2$ concentration in tear and corneal epithelium samples was measured using the Amplex Red $H_2O_2$/peroxidase assay kit (Invitrogen, Carlsbad, CA). Total antioxidant capacity was determined using the FRAP antioxidant assay kit (Beyotime, Shanghai, China). The GSH and GPX were detected using the total GSH assay kit (Beyotime, Shanghai, China) and total GPX assay kit (Beyotime, Shanghai, China), respectively.

### Topical L-glutathione treatment
Two percent L-glutathione (G4251, Sigma-Aldrich, St Louis, MO) eye drops were freshly made with PBS before treatment. Two microliters of 2% L-glutathione eye drops or PBS solvent control was administered to the ocular surface four times per day. The SD mice were continually treated with 2% L-glutathione eye drops in both eyes for 2 or 5 days, starting at day 8 or 5 after SD, respectively. The mice were continually in SD conditions during the treatment. In control groups, the 5-day SD mice received PBS treatment for 5 days. All the mice were sacrificed at the day 10 endpoint.

### Slit-lamp microscope evaluation and fluorescein staining
The corneal fluorescein staining was evaluated and scored as reported previously (Li et al., 2018a), and detailed protocols are provided in supplemental experimental procedures.

## H&E staining

The tissue section samples were stained with H&E solution (Sigma-Aldrich, USA) as previously described (Li et al., 2018b), and detailed protocols are provided in supplemental experimental procedures.

## The culture of TKE2 cell line and primary human limbal epithelial cells

TKE2, a murine corneal epithelial progenitor/stem cell line (Kawakita et al., 2008), was kindly gifted by Dr. Scheffer C.G. Tseng at Ocular Surface Center, Florida. TKE2 cells were cultured in a defined keratinocyte serum-free medium (D-KSFM) (Life Technologies Corporation, Carlsbad, CA) supplemented with 10 ng/mL epidermal growth factor (EGF) and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a 5% $CO_2$ incubator.

hLECs were isolated from donor corneas (Eye Bank Association of America) following treatment with liberase (Roche, Basel, Switzerland) and TriplE protease (LifeTech, USA). The cells were then cultured in corneal epithelial cell basal medium (PCS-700-030, ATCC) with Corneal Epithelial Cell Growth Kit (PCS-700-040, ATCC). Cells from passage 1 and 2 were used in this study.

## Cell proliferation assay

Cell proliferation assay was performed using Cell Counting Kit-8 (CCK8, Beyotime, Shanghai, China), and detailed protocols are provided in supplemental experimental procedures.

## EdU injection and detection

EdU incorporation into DNA was detected using the Click-iT EdU Alexa Fluor Imaging Kit (C10337, Thermo Fisher Scientific, MA), and detailed protocols are provided in supplemental experimental procedures.

## RNA isolation and real-time qRT-PCR analysis

Murine corneal epithelial cells were treated with TRIzol (Thermo Fisher Scientific, USA) to extract total RNA. Real-time PCR was performed using a SYBR Premix Ex Taq Kit (TaKaRa, Shiga, Japan). The primers used to amplify specific gene products are listed in Table S2. The results of relative qPCR were analyzed using the comparative threshold cycle (Ct) method and normalized to *β-actin* expression as an endogenous reference. To determine the positive expression of *Muc5ac* mRNA in cornea, ΔCt (Ct of *Muc5ac* minus Ct of *β-actin*) is less than 16 is considered positive.

## Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Li et al., 2018a). Detailed protocols are provided in supplemental experimental procedures.

## Western blot analysis

Western blot was performed using a protocol as previously described (Tang et al., 2018), and detailed protocols are provided in supplemental experimental procedures.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). All descriptive data are reported as mean ± SEM. Data are representative of two or three independent experiments. Comparisons among three or more groups were performed by one-way ANOVA test. The rate of *Muc5ac*-positive expression in cornea between groups was compared by Fisher's exact test, and $p < 0.05$ was considered statistically significant.

## Data and code availability

The accession number for the RNA-seq data reported in this paper is PRJNA819960 at the NCBI SRA database.

The authors confirm that the data supporting the findings of this study are available within the article. All raw data are available from the corresponding author on request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2022.03.017.

## AUTHOR CONTRIBUTIONS

L.S. and L.W. design the experiments. L.S., T.L., Z.J., Y.Y., and W.J. performed experiments and interpreted data. C.Y., L.Z., R.P., H.J., and L.W. interpreted data. L.S., R.P., A.S., Y.J., and L.W. wrote the manuscript. All authors critiqued and approved the manuscript.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

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