THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 32, pp. 16519–16529, August 5, 2016

Effect of Hypoxia-regulated Polo-like Kinase 3 (Plk3) on Human Limbal Stem Cell Differentiation*

Received for publication, March 4, 2016, and in revised form, June 8, 2016 Published, JBC Papers in Press, June 8, 2016, DOI 10.1074/jbc.M116.725747

Ling Wang[‡], Sheyla González[§], Wei Dai[¶], Sophie Deng[§], and Luo Lu[‡1]

From the [‡]Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, Torrance, California 90502, the [§]Department of Ophthalmology, Julie Stein Eye Institute, David Geffen School of Medicine, University of California, Los Angeles, California 91020, and the [¶]Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987

Hypoxic conditions in the cornea affect epithelial function by activating Polo-like kinase 3 (Plk3) signaling and the c-Jun·AP-1 transcription complex, resulting in apoptosis of corneal epithelial cells. Hypoxic stress in the culture conditions also regulates limbal stem cell growth and fate. In this study, we demonstrate that there is a differential response of Plk3 in hypoxic stress-induced primary human limbal stem (HLS) and corneal epithelial (HCE) cells, resulting in different pathways of cell fate. We found that hypoxic stress induced HLS cell differentiation by down-regulating Plk3 activity at the transcription level, which was opposite to the effect of hypoxic stress on Plk3 activation to elicit HCE cell apoptosis, detected by DNA fragmentation and TUNEL assays. Hypoxic stress-induced increases in c-Jun phosphorylation/activation were not observed in HLS cells because Plk3 expression and activity were suppressed in hypoxia-induced HLS cells. Instead, hypoxic stress-induced HLS cell differentiation was monitored by cell cycle analysis and measured by the decrease and increase in p63 and keratin 12 expression, respectively. Hypoxic stress-induced Plk3 signaling to regulate c-Jun activity, resulting in limbal stem cell differentiation and center epithelial apoptosis, was also found in the corneas of wild-type and Plk3$^{-/-}$-deficient mice. Our results, for the first time, reveal the differential effects of hypoxic stress on Plk3 activity in HLS and HCE cells. Instead of apoptosis, hypoxic stress suppresses Plk3 activity to protect limbal stem cells from death and to allow the process of HLS cell differentiation.

The corneal epithelial layer forms the first defense barrier to prevent environmental and biological assaults to damage the eye structures behind it. However, the protective function of the corneal epithelial layer can be damaged by several pathological conditions. Hypoxic stress is one of the leading pathological conditions that cause neovascularization, apoptosis, and attenuation of re-epithelialization in the cornea (1–5). Hypoxia-induced cellular responses, including activation of signaling events and genes, are important for the control of cell cycle progression and apoptosis (6–13). It has been reported that hypoxic conditions can induce proliferation and differentiation of various stem cells. Thus, hypoxic stress-induced cellular responses determine cell fate and healing of hypoxia-affected organs, including the cornea (14, 15). However, one of the questions that remained to be answered is how hypoxia induces different responses in stem/progenitor cells from other somatic cells, resulting in a different cell fate. To address this question, experiments were designed in our study to understand the differential effects of hypoxic stress on determinations of human limbal stem and corneal epithelial cell fate.

We have reported previously that hypoxia induces the activation of a specific Polo-like kinase 3 (Plk3) pathway in corneal epithelial and MEF[2] cells to phosphorylate downstream hypoxia-regulated PTEN, Hif-1, AP-1, p53, and H2XA, resulting in altered cell cycle progression and switching on of apoptotic responses (16–20). Plk3 is one of the four members in the Polo-like kinase family in mammalian cells, which share high homologies with *Drosophila* Polo kinases (21–24). The kinase domain the at N terminus of Plk3 phosphorylates serine and threonine residues of downstream proteins, and the other Polo-box domain at the C terminus binds to interactive proteins. As a multifunctional protein, Plk3 kinase activity and its subcellular distribution undergo substantial changes in abundance following cell cycle progression. Recent reports indicate that Plk3 is involved in regulating a variety of molecular and intracellular events that include DNA damage responses, cell cycle controls, and apoptosis (25, 26).

In corneal epithelial cells, one of the important signaling components is that the Plk3-phosphorylated c-Jun in the pathway plays an important role in forming the AP-1 complex and in the control of cell fate when the cells are challenged by various stresses to affect corneal epithelial wound healing (17, 27). Upon treating cells with hypoxic stresses, the AP-1 transcription complex is activated by extracellular stimulation mainly through eliciting MAP kinase cascades, namely the JNKs and the p38 MAPKs. Composition, regulation, and function of the AP-1 complex are different depending on the cellular context and activation of various MAP kinases. In corneal epithelial cells, activation of JNK and p38 result in increases in cell mobility and apoptosis (28–31). We found that Plk3 is involved in UV irradiation- and hypoxia-induced cell death by activation of c-Jun in corneal epithelial cells (17, 27), revealing that Plk3 is a

* This study was supported by National Institutes of Health Grants EY022364 (to L. L.), EY EY021797 (to S. D.), and CA 090658 (to W. D.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] To whom correspondence should be addressed: Dept. of Medicine, David Geffen School of Medicine at UCLA, 1124 W. Carson St., H-H Bldg., Torrance, CA 90502. Tel.: 310-781-1404; Fax: 310-781-9065; E-mail: lluou@ucla.edu.

[2] The abbreviations used are: MEF, mouse embryonic fibroblast; HCE, human corneal epithelial; HLS, human limbal stem; Ctrl, control.

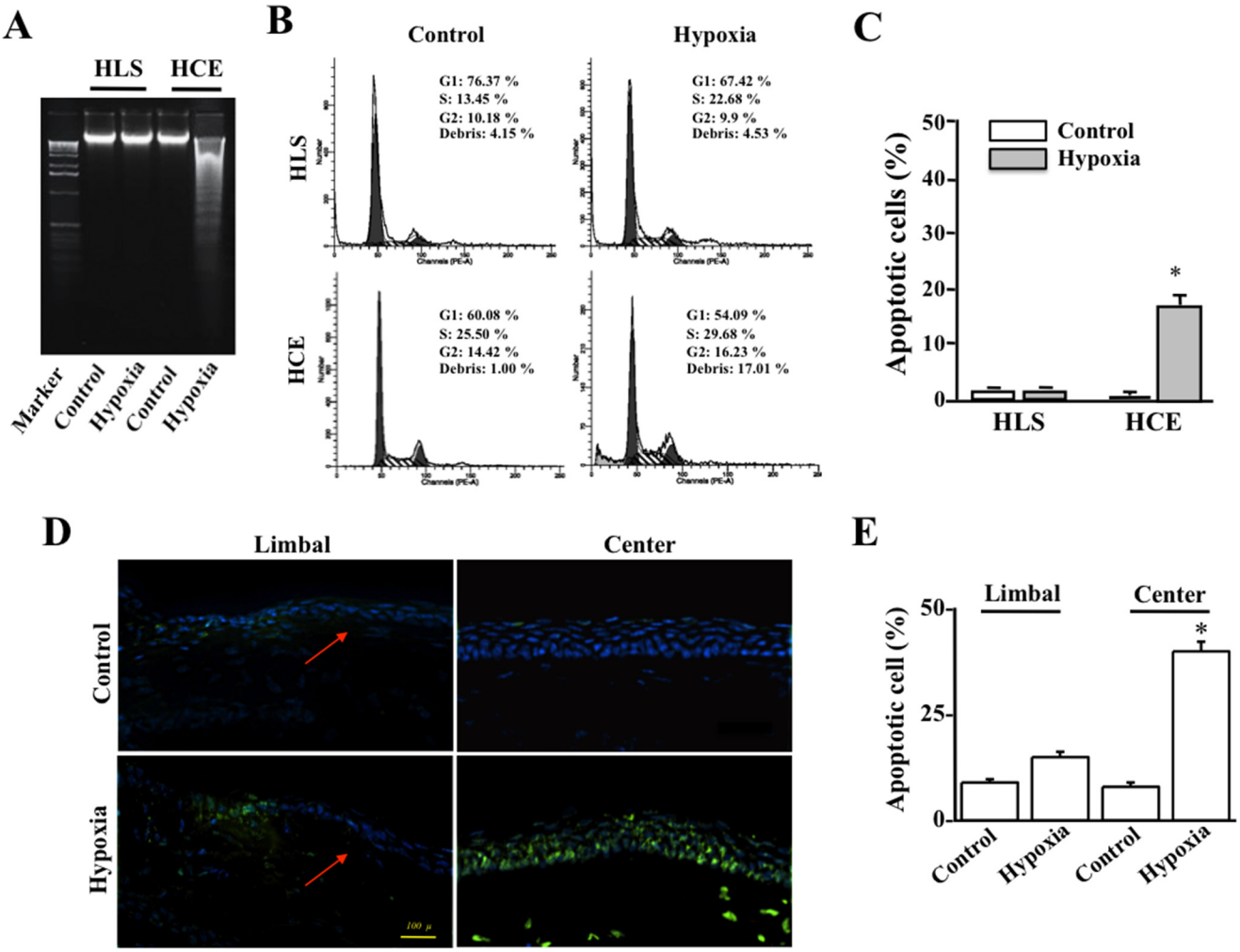

FIGURE 1. **Effect of hypoxic stress on human limbal stem and corneal epithelial cell viability.** *A*, detection of hypoxia-induced apoptosis by DNA fragmentation in primary HLS and HCE cells. *B*, effect of hypoxia on cell cycle progression in HLS and HCE cells. *C*, comparison of hypoxic stress-induced apoptosis in HLS and HCE cells. *D*, detection of hypoxic stress-induced apoptosis in the limbal region and center of the corneas of mice by TUNEL assay. The *arrows* indicate the limbal region of the cornea. *E*, statistical analysis was performed to analyze the percentage of apoptotic cells in the limbal region and in the central corneal epithelial layer. $n = 3$; *, $p < 0.05$.

newly recognized component in signaling pathways to transmit extracellular stress signals and to regulate c-Jun and ATF-2 in the AP-1 complex in addition to the existing kinase cascade pathways.

During the corneal wound healing process, corneal stem/progenitor cells located in the limbal region of the cornea migrate along the basement membrane toward the center region to replace terminally differentiated corneal epithelial cells and to repair the damaged corneal surface. It has been found that hypoxic conditions in the cornea can promote the process of corneal epithelial wound healing. However, the detailed mechanism involving hypoxia-induced limbal stem cell migration, proliferation, and differentiation is still largely unknown. In this study, we demonstrate that hypoxic conditions failed to induce human and mouse limbal stem cell apoptosis because Plk3 is suppressed, and hypoxia-sensitive target proteins further downstream, such as Hif-1 and c-Jun, are not activated in limbal stem cells. The results of this study suggest that the hypoxic endurance of limbal stem cells allows the cells to differentiate under hypoxic conditions.

Results

Effects of Hypoxic Stresses on Corneal Limbal Stem and Epithelial Cell Variability—Plk3 activity is one of the major regulators to mediate cellular responses to various stresses, including hypoxic stimulation pressure, during the wound healing process (17). Hypoxia/reoxygenation stress-induced increases in Plk3 kinase activity subsequently contribute to apoptosis in human corneal epithelial cells. In this study, application of hypoxic stress (1% oxygen) to human corneal epithelial (HCE) cells induced apoptosis detected by DNA fragmentation, which is consistent with the previous observation. However, hypoxic stress failed to induce primary human limbal stem/progenitor (HLS) cell death (Fig. 1*A*). Hypoxia-induced changes in cell cycle progression were analyzed using flow cytometry, demonstrating that hypoxic stress induced a significantly large amount of cell death in HCE cells (Fig. 1, *B* and *C*). Hypoxic stress-induced apoptosis in the mouse corneal epithelial layer was analyzed by TUNEL staining (Fig. 1*D*). In mouse corneas, there were stronger hypoxic stress-induced apoptotic effects on

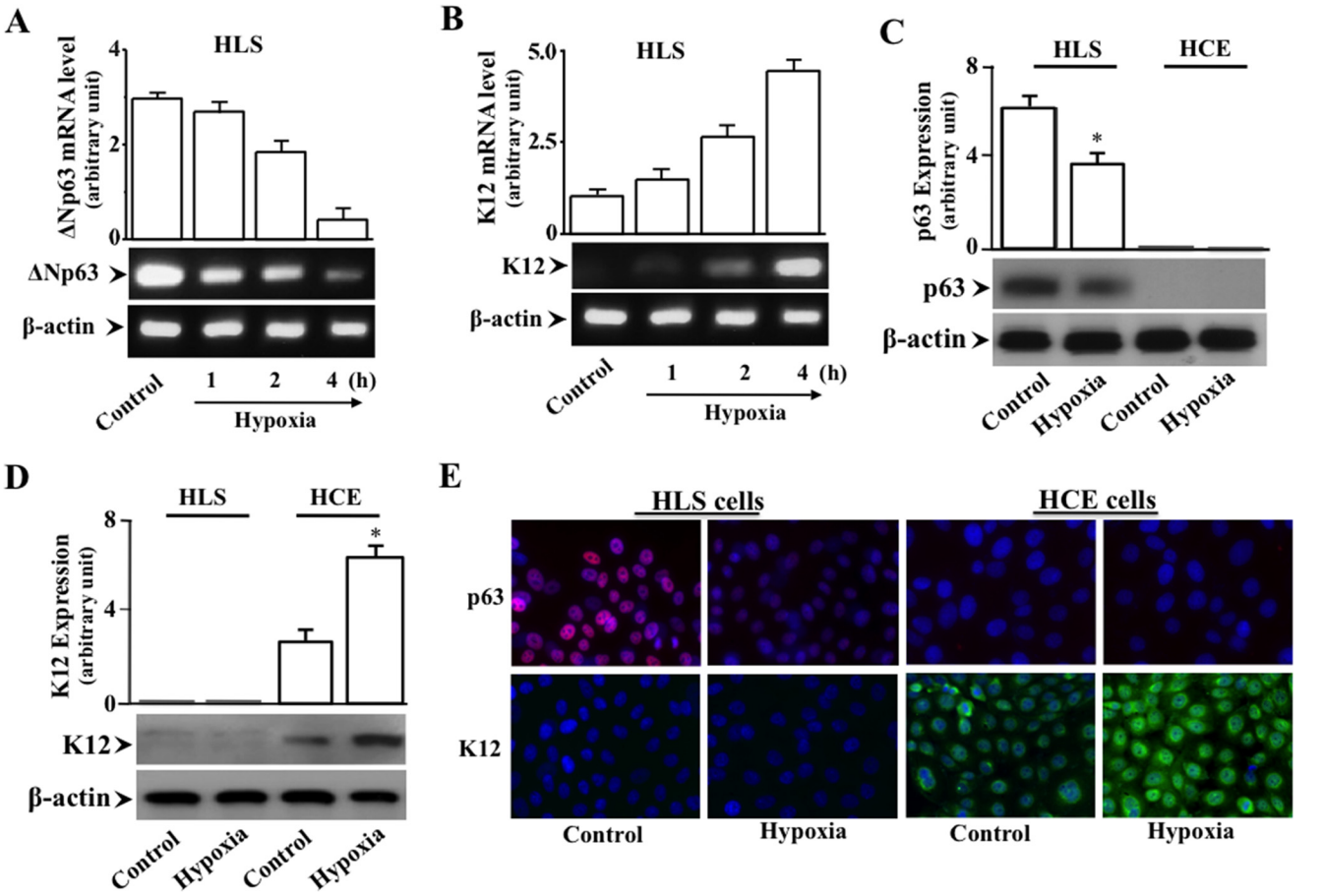

FIGURE 2. **Effect of hypoxic stress on p63 and K12 expression.** *A*, time course of changes in p63 expression in hypoxic stress-induced HLS cells. *B*, K12 expression following a time course in hypoxic stress-induced HLS cells. *C*, comparison of hypoxic stress-induced p63 expression by Western blotting analysis between HLS and HCE cells. *D*, comparison of hypoxic stress-induced p63 and K12 expression by Western blotting analysis between HLS and HCE cells. *E*, hypoxia-induced changes in expression of p63 and K12 detected by immunostaining in HLS and HCE cells, respectively. *, $p < 0.05$ (statistical significance of p63 and K12 expression in the absence and presence of hypoxic stimulation ($n = 4$).

center corneal epithelial cells (with green nuclear staining). Hypoxic stress-induced apoptosis detected by TUNEL staining was significantly higher than that found in the limbal region (Fig. 1*E*). Our results demonstrate firm evidence that there are significant differences in hypoxia-induced apoptotic responses between HLS and HCE cells.

Hypoxic Stress-induced Changes in HLS and HCE Cell Markers—As shown in different studies, p63 and keratin 12 (K12) proteins have significantly higher expression in human limbal stem and center corneal epithelial cells, respectively. To understand the effects of hypoxic stress on human limbal stem cell fate, HLS cells were induced by exposure of the cells to hypoxic conditions from 1–4 h, and mRNAs were extracted from these cells at each time point along with control HLS cells. Our results showed that p63 mRNA expression was highly expressed in control HLS cells and gradually decreased following the hypoxic exposure time course to reach the lowest point in 4 h (Fig. 2*A*). In contrast, expression of K12 mRNA was gradually increased following a hypoxic exposure time course to reach the highest point at 4 h (Fig. 2*B*). Hypoxic stress-induced expression of p63 and K12 in HLS and HCE cells was verified and compared by Western blotting analysis. Expression of the p63 and K12 proteins was found in HLS and HCE cells, respectively. Hypoxic stress induced a significant decrease in p63 expression in HLS cells (Fig. 2*C*) and a significant increase in K12 expression in HCE cells (Fig. 2*D*). Immunostaining experiments were performed to further detect expression of p63 and K12 in HLC and HCE cells with or without stimulation of hypoxic stress (Fig. 2*E*). Consistent changes in RT-PCR and Western blotting results demonstrated that hypoxic stress induced decreases in p63 expression and increases in K12 expression in HLS and HCE cells, respectively. The effects of hypoxia on the respective expression of p63 and K12 in HLS and HCE cells indicate that hypoxic stress induced HLS cell differentiation toward center corneal epithelial cells.

Effect of Hypoxic Stress-induced Active Plk3 on c-Jun Phosphorylation—We showed previously that hypoxic stress-induced increases in Plk3 kinase activity resulted in direct phosphorylation of c-Jun in corneal epithelial cells. In this study, the effect of hypoxic stress on Plk3 expression was detected at both mRNA and protein levels in HLS and HCE cells. It was consistent with a previous report showing that there was no change in mRNA expression upon hypoxic stress stimulation following a 4-h time course (Fig. 3*A*). However, hypoxic stress suppressed Plk3 mRNA expression in 1 h and, remarkably, in 4 h (Fig. 3*B*). Western blotting analysis was performed side by side in hypoxic stress-induced HLS and HCE cells, and hypoxic stress significantly suppressed Plk3 expression at the protein level (Fig. 3*C*).

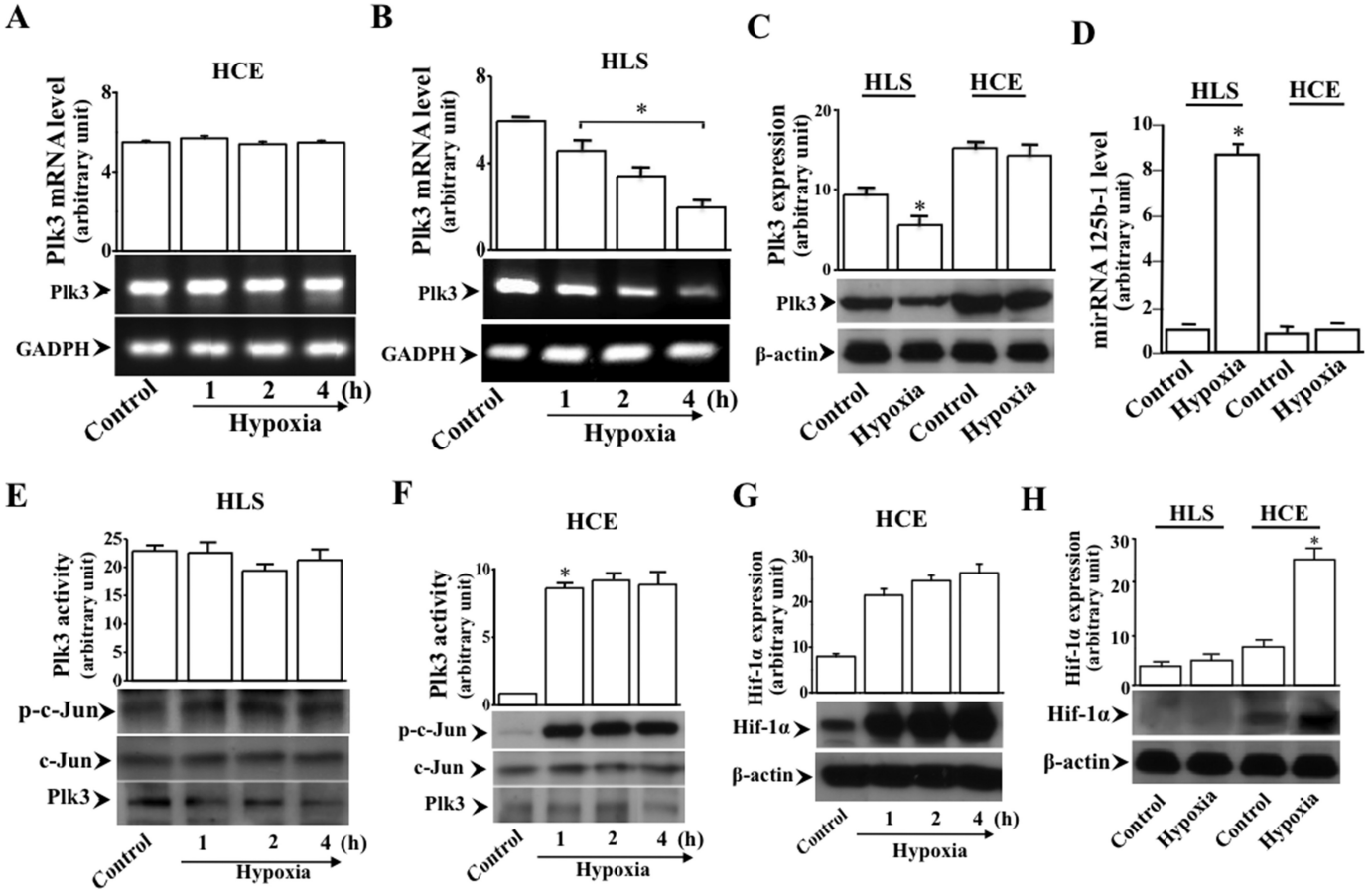

FIGURE 3. **Effect of hypoxic stress on the activation and expression of Plk3 in HLS and HCE cells.** *A*, detection of Plk3 mRNA levels in hypoxic stress-induced HCE cells. *B*, hypoxic stress-induced suppression of Plk3 mRNA expression in HLS cells. Plk3 mRNA expression in the absence and presence of hypoxic stimulation was detected by RT-PCR following a time course. *C*, hypoxic stress-induced Plk3 suppression detected by Western blotting analysis. *D*, hypoxic stress induced a high expression of Plk3-specific mirRNA125b-1 in hypoxia-induced HLS cells. *E*, effect of hypoxic stress on Plk3 kinase activity in HLS cells following a time course. *F*, hypoxic stress-induced activation of Plk3 in HCE cells following a time course. Purified c-Jun fusion protein was used as a substrate in immunocomplex kinase assays. *G*, Western blotting analysis of hypoxic stress-induced Hif-1α expression in HCE cells following a time course. *H*, hypoxic stress-induced Hif-1α expression in HLS and HCE cells detected by Western blotting analysis. *, $p < 005$ (significant difference between experimental and control groups ($n = 4$)).

The 3′ UTR sequence of Plk3 was analyzed, and there were several consensus microRNA binding sequences in this region. One of the Plk3-specific microRNAs is miRNA 125b-1, which was induced by hypoxic stress in HLS cells but was not responsive to hypoxic stress in HCE cells (Fig. 3*D*). Next, the effect of hypoxic stress on Plk3 kinase activities in HLS and HCE cells was measured by using immunocomplex kinase assays with c-Jun fusion protein as a substrate. Hypoxic stimulation did not affect Plk3 activity (no change in phospho-c-Jun level) in HLS cells (Fig. 3*E*). Hypoxic stress induced a remarkable increase in c-Jun phosphorylation, indicating that hypoxic stress activated Plk3 in HCE cells (Fig. 3*F*). The effect of hypoxic stress on Plk3 activation in HCE cells was observed in parallel to an expression of Hif-1α following a time course (Fig. 3*G*). In HCE cells, hypoxic stress-induced changes in both c-Jun and Hif-1α phosphorylation and expression were highly significant, respectively. In contrast, there was a lack of hypoxic stress-induced Hif-1α in HLS cells in 4 h (Fig. 3*H*). The results obtained from these experiments suggest that hypoxic stress significantly increased Plk3 activity and c-Jun phosphorylation in HCE cells but that it did not affect Plk3 activity in HLS cells. Instead, hypoxic stress suppressed expression of Plk3 in HLS cells. Hypoxia-induced, Plk3-specific microRNA expression was found in HLS cells but not in HCE cells, suggesting a mechanistic connection of suppression of Plk3 transcription in HLS cells.

Detection of Hypoxic Stress-induced Phospho-c-Jun Levels—Site-specific phospho-c-Jun was detected in hypoxic stress-induced HLS and HCE cells by using specific anti-c-Jun$^{Ser\text{-}63}$ and c-Jun$^{Ser\text{-}73}$ antibodies. Hypoxic stress induced significant increases in the phosphorylation levels of c-Jun$^{Ser\text{-}63}$ and c-Jun$^{Ser\text{-}73}$ in HCE cells but failed to induce changes in either c-Jun$^{Ser\text{-}63}$ or c-Jun$^{Ser\text{-}73}$ phosphorylation in HCE cells (Fig. 4, *A* and *B*). In addition, the effect of hypoxic stress on activation of phospho-c-Jun was also observed in cultured primary mouse corneal epithelial cells (Fig. 4*C*). Immunostaining experiments were performed in the limbal and central regions of mouse corneas to detect hypoxic stress-induced activation of phospho-c-Jun. The levels of phosphorylated c-Jun were rather low in the limbal and central regions of mouse corneas. Upon hypoxic stimulation, there were increases in phosphorylation of c-Jun in the central region of the corneal epithelial layer but not in the limbal region (Fig. 4*D*). The results from comparisons of hypoxic stress-induced limbal and corneal epithelial cells from human and mouse corneas suggest that the increased c-Jun

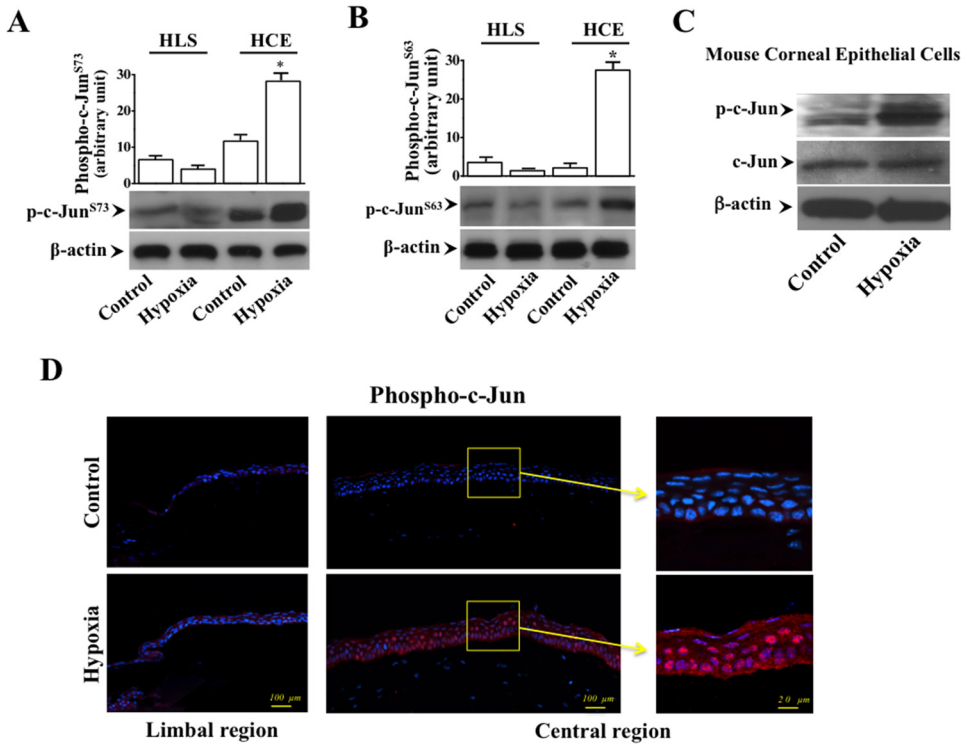

FIGURE 4. **Effect of hypoxic stress-induced c-Jun phosphorylation in HLS and HCE cells.** *A*, comparisons of hypoxic stress-induced c-Jun phosphorylation at sites of Ser-73 (c-Jun^{Ser-73}) in HLS and HCE cells. *B*, comparisons of hypoxic stress-induced c-Jun phosphorylation at sites of Ser-63 (c-Jun^{Ser-63}) in HLS and HCE cells. Statistical analysis and comparison of hypoxic stress-induced phosphorylations of c-Jun^{Ser-63} and c-Jun^{Ser-73} were performed. *C*, effect of hypoxic stress on c-Jun phosphorylation/activation in primary mouse corneal epithelial cells. *D*, effect of hypoxic stress-induced c-Jun phosphorylation/activation in the central region but not found in the limbal region of the mouse cornea. Phosphorylation of c-Jun was detected by using an anti-phospho-c-Jun antibody in immunostaining experiments. A Nikon fluorescent Ti microscope was used to capture images, and photos were labeled in different scales (20 and 100 μm). *, $p < 0.05$ (statistical significance of c-Jun activation between HLS and HCE cells in the absence and presence of hypoxic stimulation ($n = 3$)).

phosphorylation upon hypoxic stimulation only occurred in corneal epithelial cells and that the level of phospho-c-Jun in limbal stem cells was not significantly affected by hypoxic stimulation.

Effects of Hypoxia on c-Jun Activation in Plk3-suppressed Corneal Epithelial Cells—It has been shown that Plk3 mediates the phosphorylation of c-Jun, requiring interactions between Plk3 and c-Jun proteins in various stress-induced corneal epithelial cells (27). We performed further experiments to substantiate the evidence that Plk3 plays an important role in hypoxic stress-induced limbal stem and corneal epithelial cell fates by studying Plk3 activity-deficient cells and corneas from wild-type and Plk3$^{-/-}$ knockout mice. As shown in Fig. 5*A*, c-Jun and Plk3 were co-localized in HCE cells, and the interactions of c-Jun and Plk3 were markedly enhanced by hypoxic stimulation. The effect of hypoxia on c-Jun phosphorylation was significantly diminished when HCE cells were transfected with a dominant-negative Plk3 construct termed Plk3^{K52R} (27), which competitively suppresses the endogenous Plk3 activity (Fig. 5*B*). In Plk3 mRNA knockdown HCE cells, there is a time-dependent suppression of Plk3 expression, and hypoxic stress-induced phosphorylation of both c-Jun^{Ser-63} and c-Jun^{Ser-73} was decreased (Fig. 5*C*).

To further study the relationship between hypoxic stress-induced Plk3 activity and c-Jun phosphorylation in the corneal epithelium, we compared the effect of hypoxic stress on c-Jun phosphorylation in the corneas of wild-type and Plk3$^{-/-}$ knockout mice. We found, in wild-type mouse corneas, that hypoxic stress induced c-Jun phosphorylation in the center region and that there was a lack of activity of c-Jun phosphorylation in the limbal region as detected by immunostaining (Fig. 5*D*, *left panels*). In corneas of Plk3$^{-/-}$ knockout mice, there was little staining activity of c-Jun phosphorylation in response to hypoxic stimulation in either the limbal or center region of the epithelial layer, indicating that activation of Plk3 is required for hypoxic stress-induced c-Jun phosphorylation in the epithelial layer (Fig. 5*D*, *right panels*). Immunostaining study results were further verified by Western blotting analysis using primary cultured corneal epithelial cells from wild-type and Plk3$^{-/-}$ knockout mice 1 and 4 h after hypoxic stimulation, respectively (Fig. 5*E*). The effects of suppressing Plk3 on c-Jun activation further suggest that the Plk3/c-Jun signaling pathway plays a major role in hypoxic stress-induced corneal epithelium but not in the limbal region.

Effects of Altered JNK Activity and c-Jun Mutation on Hypoxia-induced c-Jun Phosphorylation—It is important to verify whether the hypoxia-induced effect of Plk3 on c-Jun phosphorylation is altered by inhibition of JNK activity and by mutation of the specific phosphorylation site of c-Jun. Inhibition of JNK expression by knockdown of JNK mRNAs with JNK-specific siRNA had no effect on hypoxia-induced phosphorylation of c-Jun^{Ser-73} (Fig. 6*A*). However, suppression of JNK activity with the JNK inhibitor SP600125 (50–100 μM) effectively inhibited hypoxia-induced c-Jun^{Ser-73} phosphorylation (Fig. 6*B*). A GST-c-Jun$^{S63A/S73A}$ double mutant was used as a substrate in immunoprecipitation and kinase assay experiments, demonstrating

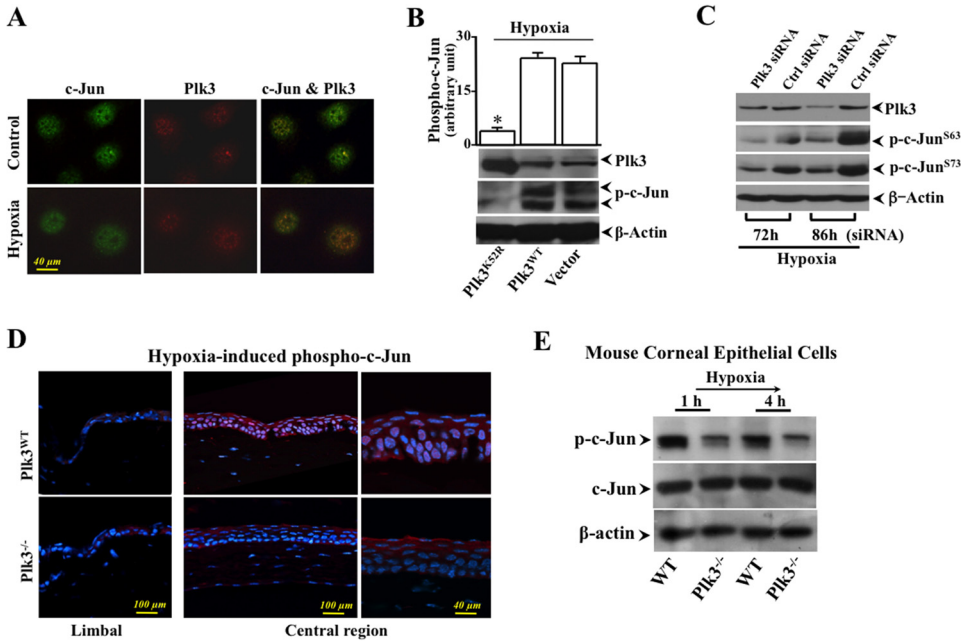

FIGURE 5. **Effect of Plk3 suppression on hypoxic stress-induced c-Jun phosphorylation.** *A*, co-localization of Plk3 and c-Jun in hypoxic stress-induced HCE cells. *B*, suppression of hypoxic stress-induced c-Jun phosphorylation by overexpression of a dominant negative $Plk3^{K52R}$ mutant. *C*, diminished hypoxic stress-induced c-Jun phosphorylation (c-$Jun^{Ser\text{-}63}$ and c-$Jun^{Ser\text{-}73}$) by knocking down Plk3 mRNA with a Plk3-specific siRNA. *D*, comparison of hypoxic stress-induced c-Jun phosphorylation in limbal and central regions of the corneas from wild-type and $Plk3^{-/-}$ mice. *E*, detection of hypoxic stress-induced c-Jun phosphorylation primary mouse corneal epithelial cells isolated from wild-type and $Plk3^{-/-}$ mice. Nuclei of corneal epithelial cells were visualized by DAPI staining in wild-type and $Plk3^{-/-}$ mouse corneas. A Nikon fluorescent Ti microscope was used to capture photos that were labeled in different scales of 40 and 100 μm.

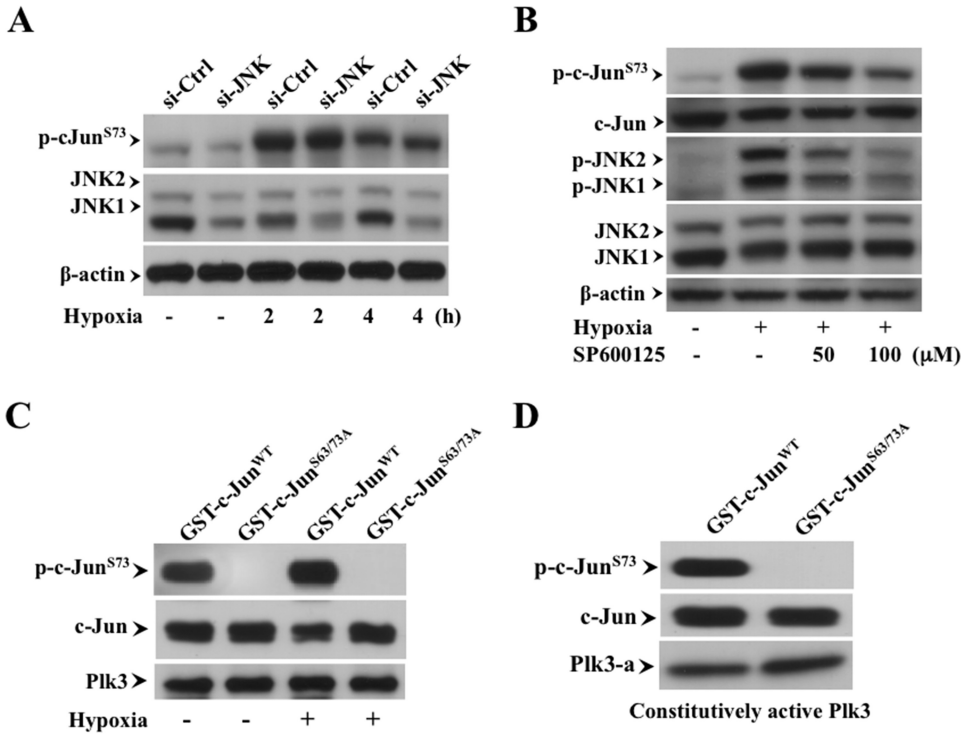

FIGURE 6. **Effect of suppression of JNK and c-Jun mutations on hypoxia-induced c-Jun activity.** *A*, effect of suppression of JNK mRNA with JNK-specific siRNA on hypoxia-induced c-Jun phosphorylation. JNK activity was suppressed by transfection of adeno-JNK-siRNA in HCE cells. *B*, effect of inhibiting JNK activity with SP600125 on hypoxia-induced c-Jun phosphorylation. *C*, effect of immunoprecipitated Plk3 from hypoxia-induced HCE cells on phosphorylation of c-Jun and the c-$Jun^{S63A/S73A}$ mutant. *D*, phosphorylation of c-Jun and the c-$Jun^{S63A/S73A}$ mutant catalyzed by purified constitutively active Plk3 *in vitro*. The mutant GSTc-$Jun^{S63A/S73A}$ was generated by replacing serine 63 and serine 73 with alanine. The GST-c-Jun fusion protein was purified by a GST fusion protein purification kit.

that hypoxia-induced active Plk3 in HCE cells could not activate phosphorylation-deficient c-$Jun^{S63A/S73A}$ (Fig. 6*C*). In a kinase assay using constitutively active Plk3 (Plk3a), Plk3a failed to phosphorylate the c-$Jun^{S63A/S73A}$ mutant (Fig. 6*D*). Our data suggest that the effect of Plk3 activation on c-Jun phosphorylation in response to hypoxic stress is specific in HCE cells.

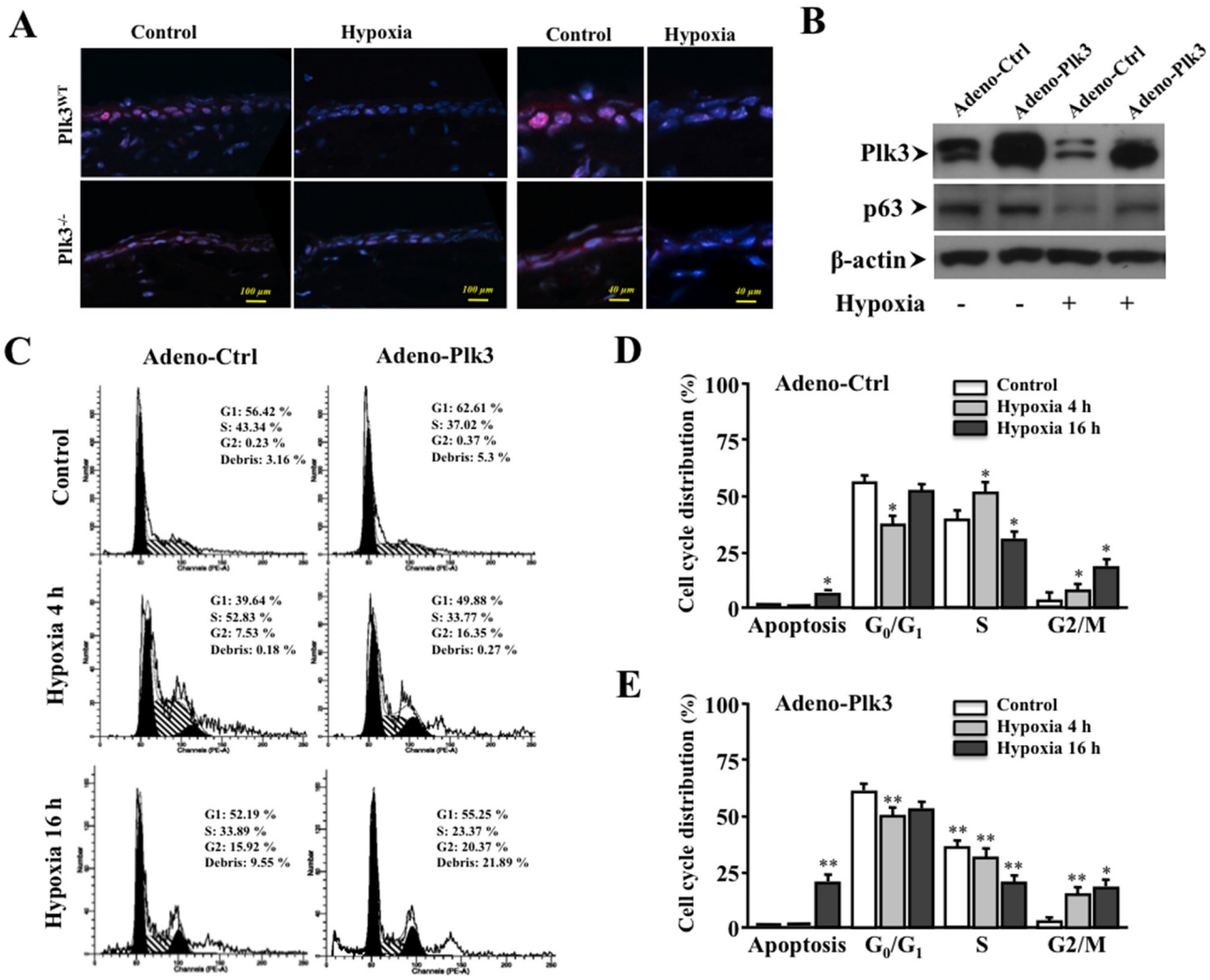

FIGURE 7. **Effect of Plk3 enhancement on hypoxic stress-induced c-Jun phosphorylation.** *A*, hypoxic stress-induced suppression of p63 in the limbal region of wild-type and Plk3$^{-/-}$ mouse corneas. Immunostaining photos were taken by a Nikon fluorescent Ti microscope in different scales of 40 and 100 μm. *B*, hypoxic stress-induced suppression of p63 detected by Western blotting analysis in adeno-control and adeno-Plk3 infected HLS cells. *C*, cell cycle analysis of hypoxic stress-induced HLS cells at 4 and 16 h. HLS cells were infected with adeno-control and adeno-Plk3 constructs and analyzed by using flow cytometry. *D*, analysis of hypoxic stress-induced alterations of cell cycle distribution and apoptosis in adeno-control-infected HLS cells. *E*, analysis of hypoxic stress-induced alterations of cell cycle distribution and apoptosis in adeno-Plk3-infected HLS cells. * and **, $p < 0.05$ (statistical significance in the absence and presence of hypoxic stimulation in adeno-control-infected cells and between adeno-control- and adeno-Plk3-infected cells, respectively ($n = 4$)).

Effects of Plk3 Activity on Hypoxia-induced Limbal Cell Fate—The effect of hypoxic stress on p63 expression was examined in human limbal stem cells that were infected with an adenoviral vector and adenoviral Plk3 constructs in the absence and presence of hypoxic stimulation. Hypoxia induced a decrease in p63 expression in both control and Plk3-overexpressed cells (Fig. 7*A*). The effect of hypoxic stress on p63 expression was also detected in the limbal region of mouse corneas obtained from wild-type and Plk3$^{-/-}$-deficient mice by using immunostaining (Fig. 7*B*). Decreased expression of p63 in hypoxia-induced human and mouse limbal cells without being affected by changes in Plk3 activity suggests that hypoxic stress induced limbal cell differentiation and that p63 expression was not directly regulated by Plk3 activity. The effect of Plk3 alteration on hypoxic stress-induced human limbal stem cell fate was analyzed at 4 and 16 h after hypoxic stimulation by using flow cytometry (Fig. 7*C*). Overexpression of Plk3 in human limbal stem cells significantly decreased and increased hypoxia-induced cell populations in S and G_2/M phases, respectively (Fig. 7, *D* and *E*). However, there was also a significantly increased apoptotic cell population in Plk3-overexpressed human limbal stem cells after exposure to hypoxic conditions for 16 h. The results indicate that hypoxia-induced differentiation in human limbal stem cells requires suppression of Plk3 activity to apoptosis under hypoxic stress conditions.

Effect of Plk3 on Hypoxia-induced Apoptosis in Plk3$^{-/-}$ mouse Corneal Epithelial and MEF Cells—The effect of hypoxia on apoptosis was studied in the cornea and mouse embryonic fibroblasts (MEFs) of Pk3$^{-/-}$ and Plk3^{WT} mice in the absence and presence of Plk3 activity, respectively. Hypoxia-induced corneal epithelial cell apoptosis was compared in corneas obtained from Plk3^{WT} and Plk3$^{-/-}$-deficient mice (Fig. 8*A*). There was a significantly reduced apoptotic response in hypoxia-induced corneas from Plk3$^{-/-}$ mice (Fig. 8*B*). However, hypoxia-induced apoptosis was not observed in the limbal region of corneas of either Plk3^{WT} or Plk3$^{-/-}$ mice (data not

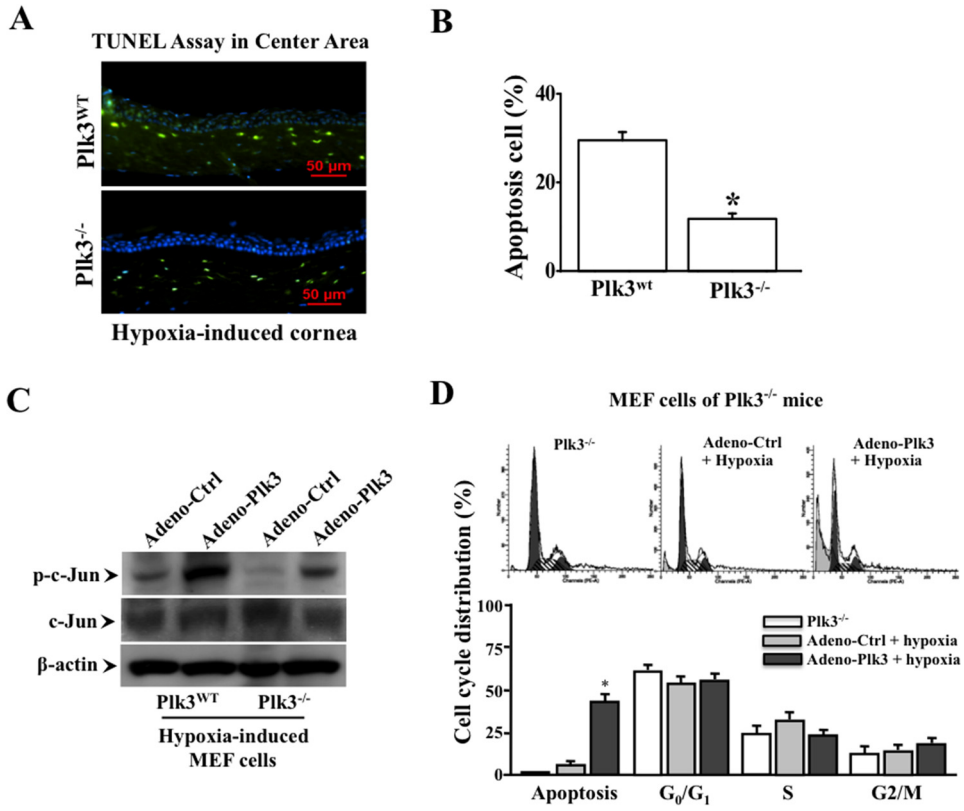

FIGURE 8. **Hypoxia-induced apoptosis in corneas and MEF cells obtained from Plk3^{WT} and Plk3$^{-/-}$ mice.** A, hypoxia-induced central corneal epithelial cell apoptosis detected by TUNEL assay in Plk3^{WT} and Plk3$^{-/-}$ mice. *B*, statistical significance of hypoxia-induced apoptosis between corneas of Plk3^{WT} and Plk3$^{-/-}$ mice. *C*, hypoxia-induced c-Jun activation of Plk3$^{-/-}$, adeno-Ctrl, and adeno-Plk3 MEF cells. *D*, hypoxia-induced apoptosis in Plk3$^{-/-}$, adeno-Ctrl, and adeno-Plk3 MEF cells. MEF cells obtained from Plk3$^{-/-}$ mice were used as the basic controls, and MEF cells obtained from Plk3$^{-/-}$ mice were infected with adeno-vector (*adeno-Ctrl*) and cDNA encoding full-length Plk3 (*adeno-Plk3*) for the experimental group control and for the Plk3 restoration study, respectively. *, $p < 0.05$; $n = 3$.

shown). In MEF cells obtained from Plk3$^{-/-}$ mice, hypoxic stress failed to induced c-Jun activation. However, c-Jun phosphorylation was markedly increased in Plk3^{WT} and Plk3-overexpressed Plk3$^{-/-}$ MEF cells in which Plk3 activity was restored (Fig. 8*C*). The effects of hypoxia-induced changes on cell cycle progression were studied in adeno-vector (adeno-Ctrl) and Plk3-transfected (adeno-Plk3) MEF cells isolated from Plk3$^{-/-}$ mice. There was a significant increase in the apoptotic population of Plk3-overexpressed (adeno-Plk3) Plk3$^{-/-}$ MEF cells (Fig. 8*D*). Results obtained from Plk3$^{-/-}$ corneas and MEF cells provided more evidence showing that Plk3 plays a significant role in the mediation of hypoxic stress in various cells.

Discussion

The epithelial layer on the surface of the cornea plays important roles in the defense against environmental stresses, including biohazards, UV irradiation, and hypoxic and hyperosmotic stresses. During the corneal epithelial self-renewal and wound healing processes, corneal limbal stem cells differentiate to become transient amplifying cells (progenitor cells) that migrate toward the center region of the cornea along the basement membrane to become and replace surface terminal differentiated epithelial cells (32). If there is any damage in the limbal region, it can result in permanent scaring of the cornea, and a corneal transplant may be required. The question that remained to be answered is what is the driving force or factors that direct limbal stem/progenitor cells to migrate toward the center region to execute the wound healing function. In this study, we reveal for the first time that there are different cellular responses to hypoxic stress between limbal stem and corneal epithelial cells in humans and mice, involving hypoxic stress-induced Plk3/c-Jun signaling, resulting in a distinct path of cell fates.

Recent studies found that hypoxic stress (3–5% oxygen in the culture conditions) can result in increased proliferation of limbal stem cells (14, 15). More restricted hypoxic condition (1% oxygen) trigger corneal epithelial apoptosis by activating Plk3 kinase to directly phosphorylate c-Jun and to elicit JNK signaling cascades, resulting in activation of c-Jun/AP-1 in corneal epithelial cells (17). In this study, we found that HLS cells were more tolerant of harsh hypoxic conditions compared with HCE cells and that they underwent hypoxic stress-induced differentiation instead of apoptosis. Interestingly, Plk3 expression in hypoxia-induced HLS cells was altered by hypoxic stimulation and down-regulated at the transcription level (Fig. *3*). This is opposite to the effect of hypoxic stress on increasing Plk3 activity in HCE cells. In these experiments, Hif-1 expression increased in HCE cells with hypoxia treatment, indicating that the hypoxia condition is successful or proved. However, Hif-1 was not expressed in HLS cells when these cells were treated side-by-side with HCE cells in the hypoxic chamber up to 4 h. This observation provides further supporting evidence for hypoxia-induced down-regulation of Plk3 in HLS

cells because Hif-1 is one of the downstream components in the Plk3 signaling pathway (19, 20).

Plk3 is a multifunctional protein kinase that is activated in response to various environmental stresses (21). In corneal epithelial cells, Plk3 kinase is activated to strongly phosphorylate c-Jun in HCE cells without altering the Plk3 expression level. As shown in this study, Plk3-mediated c-Jun phosphorylation in hypoxic stress-induced corneal epithelial cells showed that the time course of c-Jun phosphorylation is consistent with the time course of Hif-1 activation from 0.5–4 h. The effect of hypoxic stress on Plk3 activity in HLS cells has not been reported previously in published literature. Our results reveal, for the first time, that Plk3 expression and kinase activity are suppressed by hypoxic stress in HLS cells. In addition, the effect of hypoxic stress on c-Jun phosphorylation/activation was verified in corneal stem/progenitor cells in the limbal region by comparing wild-type and $Plk3^{-/-}$ knockout mouse corneas with immunostaining studies (Figs. 3–5).

Keratin 12 and p63s have been identified as the relative specific markers in human corneal epithelial and limbal stem cells, respectively. It has been shown that a significantly higher level of p63 found in limbal stem cells is important in the maintenance of pluripotency of these cells and that it plays an important role in the development of corneal epithelia (33, 34). In the study, human and mouse limbal stem/progenitor cells were carefully monitored by detection of p63 expression levels and compared with center corneal epithelial cells. Hypoxic stress-induced apoptosis of human and mouse corneal epithelial cells and MEF cells was determined by DNA fragmentation and TUNEL staining, respectively (Figs. 1 and 8). We found that there is a close correlation between Plk3 activity and hypoxia-induced apoptosis in corneal epithelial cells. In fact, hypoxic stress has much less of an effect on induction of apoptosis in HLS, $Plk3^{-/-}$ HCE, and $Plk3^{-/-}$ MEF cells. In addition, differentiation of primary human corneal epithelial cells was indicated by detection of K12 expression, which is known as a lineage-specific differentiation marker for human corneal epithelium (35, 36). With these specific studies, we observed dramatic transitional changes in hypoxic stress-induced limbal stem cell differentiation and corneal epithelial apoptosis. In fact, we were able to demonstrate that hypoxic stress-induced limbal stem cell differentiation is accompanied by a reduction in p63 expression and that it correlates with K12 expression in primary human corneal epithelial cells. In the study, cell cycle analysis of HLS and HCE cells was performed in the absence and presence of hypoxic stress. Hypoxic stress induced a significant increase in apoptosis exclusively in HCE cells within 4 h of treatment, while no change was observed in HLS cells. Overexpression of Plk3 in HLS cells resulted in significant cell population changes in S and G_2/M phase 4 h after hypoxic treatment (Fig. 7). In addition, overexpression of Plk3 resulted in significant increases in the apoptotic cell population in 4 and 16 h of hypoxic stress-induced HLS cells. Thus, there is no doubt that our findings in this study provide important evidence establishing that hypoxic stimulation activates Plk3 and plays a critical role in mediating c-Jun activation and apoptosis in corneal epithelial cells. The other finding of this study is that hypoxia-induced c-Jun phosphorylation was not observed in HLS cells (data not shown), which may result from hypoxic stress significantly suppressing Plk3 expression because we found that there was a highly expressed Plk3-specific microRNA in hypoxia-induced HLS cells (Fig. 3*D*). Taken together, hypoxic stimulation results in HLS cell differentiation, which can be very important to provide a leading signal and driving force for limbal stem/progenitor cells to engage in corneal epithelial self-renewal and wound healing.

Experimental Procedures

Cell Culture and Treatment

HLS Cell Culture—Human sclerocorneal tissues were obtained from the Illinois Eye Bank (Watson Gailey, Bloomington, IL) and the Lions Eye Institute for Transplant and Research (Tampa, FL) from 20- to 70-year-old healthy donors. Human tissue was handled in accordance with the tenets of the Declaration of Helsinki. The experimental protocol was exempted by the University of California Los Angeles Institutional Review Board. The tissues were preserved in Optisol^{TM} (Chiron Ophthalmics, Inc., Irvine, CA) at 4 °C. The death-to-preservation time was less than 10 h. After removal of the iris, endothelium, conjunctiva, and Tenon capsule, the sclerocorneal rim tissue was incubated in 2.4 units/ml of dispase II (Roche) at 37 °C for 2 h in DMEM/F-12 (Ham) medium (Life Technologies) with 5% FBS (Life Technologies). After dispase II incubation, an epithelial cell sheet was isolated by gentle scraping under the dissecting microscope and incubated with 0.25% trypsin, 1 mM EDTA (Life Technologies) for 5 min to achieve a single-cell suspension. HLS cells were cultured at a density of 300 cells/cm^2 on a monolayer of mitomycin C (Sigma-Aldrich, St. Louis, MO) and growth-arrested 3T3-J2 mouse fibroblasts (Howard Green laboratory, Harvard Medical School) at a density of 3×10^4 cm^2 in supplemental hormone epithelial medium consisting of DMEM/F-12 (Ham) medium supplemented with 5% FBS, N2 supplement (Life Technologies), 2 ng/ml epidermal growth factor (Life Technologies), 8.4 ng/ml cholera toxin (Sigma-Aldrich), 0.5 g/ml hydrocortisone (Sigma-Aldrich), and 0.5% dimethyl sulfoxide (Sigma-Aldrich) at 37 °C under 5% CO_2 for 14 days. The medium was changed every 2–3 days.

HCE Cells—Primary HCE cells were obtained by passing limbal stem cells onto a collagen/fibronectin (50/50%)-coated surface and an airlift (reduced medium level) procedure to induce differentiation. HCE cells were grown in serum-free defined keratinocyte medium (Invitrogen) in an incubator supplied with 95% air and 5% CO_2 at 37 °C. The morphological changes were closely monitored. In addition, freshly isolated primary HCE cells were also obtained by using an established explant method in the laboratory (37, 38). For experiments of hypoxic stress stimulation, HLS and HCE cells were placed in premixed gas with various oxygen concentrations from 0.5% to 3% and 5% CO_2 continuously perfused into a humidified hypoxic chamber (BioSpherix) at 37 °C. The O_2 level in the chamber was monitored by a built-in O_2 meter.

Mouse Eyeball Organ Culture

Corneas from both wild-type and $Plk3^{-/-}$ knockout mice were used in the study (39). Whole eyeballs of mice were dissected and placed in culture wells (the corneas facing up) on a

supporter made of 1% agarose and 1 mg/ml rat tail tendon collagen in minimum Eagle's medium containing 10% FBS and 1% antibiotic/antimycotic. The surface of the center cornea was covered with 2 mM glutamine, and the epithelium was exposed to the air (airlift) (40, 41). The eyes were incubated for various time points at 37 °C and 5% CO_2. For quality control, cultured mouse corneas in each batch were randomly sampled with ZO-1, Na-K-ATPase, and BrdU staining. Hypoxic induction of mouse eyeballs was performed by placing cultured eyeballs in premixed gas with various oxygen concentrations from 0.5% to 3% and 5% CO_2 continuously perfused into a humidified hypoxic chamber (BioSpherix).

Cell Cycle Analysis and Apoptosis Assays

Cell Cycle Analysis—Cell cycle analysis was performed using a flow cytometer (BD LSRII, BD Biosciences). Cells were treated with calcium (1.2 mM) and FBS (5%) following a time course or left untreated, and then the attached cells were trypsinized and fixed with 70% ethanol and 50 mM glycine. The cells were resuspended in PBS containing RNase A (100 ng/ml) and propidium iodide (25 ng/ml). Cell populations in different phases were mapped with BD FAC Diva software V6.11), and cell cycle progression was analyzed with MedFit LT™ V3.1 (Verity Software House).

Measurement of DNA Fragmentation—Cells were washed twice with PBS. Lysis buffer (200 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1% SDS, and 100 μg/ml proteinase K) was added, and cells were then incubated for 4 h at 55 °C. The nuclear lysates were extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with 0.05 volume of 5 M NaCl and 2.5 volumes of absolute ethanol, incubated overnight at −20 °C, and centrifuged at 13,000 × *g* for 10 min at 4 °C. The DNA pellet was dried and dissolved in TE buffer (10 mM Tris-HCl (pH 7.5) and 1 mM EDTA) containing 20 μg/ml RNase A and incubated for 1 h at 37 °C. The DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA samples were analyzed by electrophoresis on 1.5% agarose gels, and the results were visualized by staining with 1 μg/ml ethidium bromide.

TUNEL Experiments—To detect apoptotic cells in the corneal epithelial layer, TUNEL staining was performed using a DeadEnd™ fluorimetric TUNEL system (Promega, Madison, WI) according to the protocol of the manufacturer. Mouse corneal sections were washed in PBS and deproteinized (or permeabilized) by proteinase K (20 μg/ml) for 10 min at room temperature. Mouse corneal sections were rinsed with PBS and placed in incubation buffer containing equilibration buffer, nucleotide mixture, and rTdT enzyme at 37 °C for 1 h in the dark. Mouse corneal sections were treated with DAPI nuclear stain and proceeded to analysis using a Nikon fluorescent Ti microscope and Nikon NIS Element software.

Immunocytochemistry

Immunostaining experiments—For immunostaining experiments, mouse corneal frozen sections were fixed for 15 min in 4% paraformaldehyde and then permeabilized with PBS-0.2% Triton X-100 (PBS-T) for 30 min at room temperature. The tissues were blocked by incubation with 10% normal horse serum or 10% normal goat serum in PBS-T for 1 h at room temperature, followed by immunostaining with the corresponding antibodies. Corneal tissue slices were washed with PBS and stained with DAPI. A Nikon fluorescent Ti microscope was used to capture stained tissue. Imaging data were analyzed using a Nikon NIS Element software program.

Immunoprecipitation and Immunocomplex Kinase Assays—Human corneal epithelial cells (5×10^7) were rinsed with PBS and incubated in 1 ml of lysis buffer (20 mM Tris (pH 7.5), 137 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 10 mM sodium pyrophosphate, 25 mM glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 250 μM 4-nitrophenyl phosphate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice for 30 min. The cell lysates were spun at 13,000 × *g* for 10 min at 4 °C and incubated at 4 °C overnight with antibodies against Plk3 and other proteins, respectively. The immunocomplexes were recovered by incubation with 50 μl of 10% protein A/G-Sepharose (Santa Cruz Biotechnology). The immunocomplex beads were rinsed twice with lysis buffer and once with kinase buffer and then subjected to immunoblotting and a kinase assay. The effect of active Plk3 on catalyzing c-Jun phosphorylation was measured using immunocomplex kinase assays by incubation of immunoprecipitated Plk3 with c-Jun fusion protein in 30 μl of kinase buffer (20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 10 μM $MnCl_2$, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 20 μM ATP, and 10 μCi of [γ-^{32}P]ATP) for 30 min at 37 °C. Kinase reactions were terminated by adding an equal volume of 2× Laemmli buffer and boiling for 5 min. Equal volumes of the samples were displayed on 10–15% SDS-PAGE and visualized by exposure on x-ray films. For cold kinase assay, [γ-^{32}P]ATP was omitted from the kinase buffer. Western blotting was performed by harvesting 10^5 cells in 0.5 ml of lysis buffer. Samples were loaded into 10–15% SDS-PAGE gels and fractionated by electrophoresis. Proteins in the gel were electrotransferred to a PVDF membrane. The membrane was exposed to blocking buffer (TBS-T) for 1 h at room temperature and then incubated with the respective antibodies overnight at 4 °C. After three washes with TBS-T buffer, the membrane was incubated with HRP-linked secondary antibody for 1 h at room temperature. Expression of proteins was detected with a Western blotting detection kit (Santa Cruz Biotechnology).

Author Contributions—L. W. designed and performed most of the experiments and wrote parts of the paper. S. G. isolated human limbal stem cells and wrote parts of the paper. W. D. provided Plk3 constructs and knockout mice. S. D. provided human limbal stem cells and was a consultant for the project. L. L. was involved in designing and performing the experiments and wrote the manuscript.

References

1. Singh, N., Amin, S., Richter, E., Rashid, S., Scoglietti, V., Jani, P. D., Wang, J., Kaur, R., Ambati, J., Dong, Z., and Ambati, B. K. (2005) Flt-1 intraceptors inhibit hypoxia-induced VEGF expression *in vitro* and corneal neovascularization *in vivo*. *Invest. Ophthalmol. Vis. Sci.* **46,** 1647–1652
2. Singh, N., Jani, P. D., Suthar, T., Amin, S., and Ambati, B. K. (2006) Flt-1 intraceptor induces the unfolded protein response, apoptotic factors, and regression of murine injury-induced corneal neovascularization. *Invest. Ophthalmol. Vis. Sci.* **47,** 4787–4793

3. Cursiefen, C., Maruyama, K., Jackson, D. G., Streilein, J. W., and Kruse, F. E. (2006) Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea* **25,** 443–447
4. Mwaikambo, B. R., Sennlaub, F., Ong, H., Chemtob, S., and Hardy, P. (2006) Activation of CD36 inhibits and induces regression of inflammatory corneal neovascularization. *Invest. Ophthalmol. Vis. Sci.* **47,** 4356–4364
5. Zhang, M. C., Wang, Y., and Yang, Y. (2006) The expression of nuclear factor κ B in inflammation-induced rat corneal neovascularization. *Ocul. Immunol. Inflamm.* **14,** 359–365
6. Laderoute, K. R., Calaoagan, J. M., Gustafson-Brown, C., Knapp, A. M., Li, G. C., Mendonca, H. L., Ryan, H. E., Wang, Z., and Johnson, R. S. (2002) The response of c-jun/AP-1 to chronic hypoxia is hypoxia-inducible factor 1 α dependent. *Mol. Cell Biol.* **22,** 2515–2523
7. Nishi, H., Nishi, K. H., and Johnson, A. C. (2002) Early growth response-1 gene mediates up-regulation of epidermal growth factor receptor expression during hypoxia. *Cancer Res.* **62,** 827–834
8. Laderoute, K. R., Calaoagan, J. M., Knapp, M., and Johnson, R. S. (2004) Glucose utilization is essential for hypoxia-inducible factor 1 α-dependent phosphorylation of c-Jun. *Mol. Cell Biol.* **24,** 4128–4137
9. Seagroves, T. N., Ryan, H. E., Lu, H., Wouters, B. G., Knapp, M., Thibault, P., Laderoute, K., and Johnson, R. S. (2001) Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. *Mol. Cell Biol.* **21,** 3436–3444
10. Comerford, K. M., Cummins, E. P., and Taylor, C. T. (2004) c-Jun NH2-terminal kinase activation contributes to hypoxia-inducible factor 1α-dependent P-glycoprotein expression in hypoxia. *Cancer Res.* **64,** 9057–9061
11. Dougherty, C. J., Kubasiak, L. A., Frazier, D. P., Li, H., Xiong, W. C., Bishopric, N. H., and Webster, K. A. (2004) Mitochondrial signals initiate the activation of c-Jun N-terminal kinase (JNK) by hypoxia-reoxygenation. *FASEB J.* **18,** 1060–1070
12. Liu, H., Ma, Y., Pagliari, L. J., Perlman, H., Yu, C., Lin, A., and Pope, R. M. (2004) TNF-α-induced apoptosis of macrophages following inhibition of NF-κ B: a central role for disruption of mitochondria. *J. Immunol.* **172,** 1907–1915
13. McCloskey, C. A., Kameneva, M. V., Uryash, A., Gallo, D. J., and Billiar, T. R. (2004) Tissue hypoxia activates jnk in the liver during hemorrhagic shock. *Shock* **22,** 380–386
14. Miyashita, H., Higa, K., Kato, N., Kawakita, T., Yoshida, S., Tsubota, K., and Shimmura, S. (2007) Hypoxia enhances the expansion of human limbal epithelial progenitor cells *in vitro*. *Invest. Ophthalmol. Vis. Sci.* **48,** 3586–3593
15. O'Callaghan, A. R., Daniels, J. T., and Mason, C. (2011) Effect of subatmospheric oxygen on the culture of rabbit limbal epithelial cells. *Curr. Eye Res.* **36,** 691–698
16. Wang, L., Dai, W., and Lu, L. (2005) Ultraviolet irradiation-induced K^+ channel activity involving p53 activation in corneal epithelial cells. *Oncogene* **24,** 3020–3027
17. Wang, L., Gao, J., Dai, W., and Lu, L. (2008) Activation of Polo-like kinase 3 by hypoxic stresses. *J. Biol. Chem.* **283,** 25928–25935
18. Wang, L., Payton, R., Dai, W., and Lu, L. (2011) Hyperosmotic stress-induced ATF-2 activation through Polo-like kinase 3 in human corneal epithelial cells. *J. Biol. Chem.* **286,** 1951–1958
19. Xu, D., Yao, Y., Jiang, X., Lu, L., and Dai, W. (2010) Regulation of PTEN stability and activity by Plk3. *J. Biol. Chem.* **285,** 39935–39942
20. Xu, D., Yao, Y., Lu, L., Costa, M., and Dai, W. (2010) Plk3 functions as an essential component of the hypoxia regulatory pathway by direct phosphorylation of HIF-1α. *J. Biol. Chem.* **285,** 38944–38950
21. Dai, W. (2005) Polo-like kinases, an introduction. *Oncogene* **24,** 214–216
22. Donohue, P. J., Alberts, G. F., Guo, Y., and Winkles, J. A. (1995) Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. *J. Biol. Chem.* **270,** 10351–10357
23. Hamanaka, R., Smith, M. R., O'Connor, P. M., Maloid, S., Mihalic, K., Spivak, J. L., Longo, D. L., and Ferris, D. K. (1995) Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. *J. Biol. Chem.* **270,** 21086–21091
24. Li, B., Ouyang, B., Pan, H., Reissmann, P. T., Slamon, D. J., Arceci, R., Lu, L., and Dai, W. (1996) Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. *J. Biol. Chem.* **271,** 19402–19408
25. Conn, C. W., Hennigan, R. F., Dai, W., Sanchez, Y., and Stambrook, P. J. (2000) Incomplete cytokinesis and induction of apoptosis by overexpression of the mammalian polo-like kinase, Plk3. *Cancer Res.* **60,** 6826–6831
26. Wang, Q., Xie, S., Chen, J., Fukasawa, K., Naik, U., Traganos, F., Darzynkiewicz, Z., Jhanwar-Uniyal, M., and Dai, W. (2002) Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. *Mol. Cell Biol.* **22,** 3450–3459
27. Wang, L., Dai, W., and Lu, L. (2007) Stress-induced c-Jun activation mediated by Polo-like kinase 3 in corneal epithelial cells. *J. Biol. Chem.* **282,** 32121–32127
28. Lee, J. H., Kim, M., Im, Y. S., Choi, W., Byeon, S. H., and Lee, H. K. (2008) NFAT5 induction and its role in hyperosmolar stressed human limbal epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **49,** 1827–1835
29. Lu, L., Wang, L., and Shell, B. (2003) UV-induced signaling pathways associated with corneal epithelial cell apoptosis. *Invest. Ophthalmol. Vis. Sci.* **44,** 5102–5109
30. Kimura, K., Teranishi, S., Yamauchi, J., and Nishida, T. (2008) Role of JNK-dependent serine phosphorylation of paxillin in migration of corneal epithelial cells during wound closure. *Invest. Ophthalmol. Vis. Sci.* **49,** 125–132
31. Saika, S., Okada, Y., Miyamoto, T., Yamanaka, O., Ohnishi, Y., Ooshima, A., Liu, C. Y., Weng, D., and Kao, W. W. (2004) Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* **45,** 100–109
32. Tseng, S. C. (1989) Concept and application of limbal stem cells. *Eye* **3,** 141–157
33. Arpitha, P., Prajna, N. V., Srinivasan, M., and Muthukkaruppan, V. (2005) High expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: implications on epithelial stem cells. *Invest. Ophthalmol. Vis. Sci.* **46,** 3631–3636
34. Parsa, R., Yang, A., McKeon, F., and Green, H. (1999) Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J. Invest. Dermatol.* **113,** 1099–1105
35. Nishida, K., Adachi, W., Shimizu-Matsumoto, A., Kinoshita, S., Mizuno, K., Matsubara, K., and Okubo, K. (1996) A gene expression profile of human corneal epithelium and the isolation of human keratin 12 cDNA. *Invest. Ophthalmol. Vis. Sci.* **37,** 1800–1809
36. Tanifuji-Terai, N., Terai, K., Hayashi, Y., Chikama, T., and Kao, W. W. (2006) Expression of keratin 12 and maturation of corneal epithelium during development and postnatal growth. *Invest. Ophthalmol. Vis. Sci.* **47,** 545–551
37. Nakatsu, M. N., Ding, Z., Ng, M. Y., Truong, T. T., Yu, F., and Deng, S. X. (2011) Wnt/β-catenin signaling regulates proliferation of human cornea epithelial stem/progenitor cells. *Invest. Ophthalmol. Vis. Sci.* **52,** 4734–4741
38. Truong, T. T., Huynh, K., Nakatsu, M. N., and Deng, S. X. (2011) SSEA4 is a potential negative marker for the enrichment of human corneal epithelial stem/progenitor cells. *Invest. Ophthalmol. Vis. Sci.* **52,** 6315–6320
39. Lu, J., Wang, L., Dai, W., and Lu, L. (2010) Effect of hypoxic stress-activated Polo-like kinase 3 on corneal epithelial wound healing. *Invest. Ophthalmol. Vis. Sci.* **51,** 5034–5040
40. Xu, K. P., Li, X. F., and Yu, F. S. (2000) Corneal organ culture model for assessing epithelial responses to surfactants. *Toxicol. Sci.* **58,** 306–314
41. Ramaesh, T., Ramaesh, K., Leask, R., Springbett, A., Riley, S. C., Dhillon, B., and West, J. D. (2006) Increased apoptosis and abnormal wound-healing responses in the heterozygous $Pax6^{+/-}$ mouse cornea. *Invest. Ophthalmol. Vis. Sci.* **47,** 1911–1917