

Involvement of p27^{KIP1} Degradation by Skp2 in the Regulation of Proliferation in Response to Wounding of Corneal Epithelium

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PURPOSE. To examine the expression of the p27^{KIP1} in the normal and epithelial-scraped cornea and whether degradation of p27^{KIP1} by Skp2 is involved in the regulation of cell proliferation in response to wounding of the corneal epithelium.

METHODS. C57Bl6, p27^{KIP1}^{-/-}, Skp2^{-/-}, and Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice were examined. Normal and epithelial-scraped corneas were analyzed by immunocytochemistry using anti-p27^{KIP1} antibody. Cells in the S phase of DNA synthesis were analyzed by immunocytochemistry using anti-bromodeoxyuridine (BrdU) antibody.

RESULTS. The p27^{KIP1} was expressed in basal cells of the central and peripheral region of the cornea and limbus. This expression was not detected 24 hours after the epithelial scraping, when there were many cells in the S phase of DNA synthesis in the corneal epithelium. There were no obvious differences in the thickness and anti-BrdU staining in the corneal epithelium of p27^{KIP1}^{-/-} mice from that of control animals. Twenty-four hours after epithelial scraping in the Skp2^{-/-} mice, the corneal epithelium was thinner than in wild-type mice and had many p27^{KIP1}-positive cells and few BrdU-positive cells. In contrast, 24 hours after epithelial scraping in the Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice, the corneal epithelium was as thick as in wild-type mice and had many BrdU-positive cells.

CONCLUSIONS. These results suggest that degradation of p27^{KIP1} by Skp2 is involved in the regulation of cell proliferation in response to wounding of the corneal epithelium. (*Invest Ophthalmol Vis Sci.* 2002;43:364-370)

Wounding of the central corneal epithelium stimulates cell proliferation.¹ Corneal epithelial debridement stimulates basal cells outside the wound area to synchronously enter the cell cycle.² However, the mechanism of the regulation of cell proliferation has not been determined. Cell cycle progression is controlled by a series of kinase complexes composed of cyclins and cyclin-dependent kinases (CDKs).³ The enzymatic

activities of cyclin/CDK complexes are regulated by many mechanisms that reflect both the diversity of the signals they integrate and the central importance of their roles in cell cycle control. These regulatory mechanisms include the actions of CDK inhibitors (CKIs).^{4,5} p27^{KIP1} is one of the CKIs and the elimination of p27^{KIP1} during the late G₁ phase is required for cell cycle progression from the G₁ to S phase in various cell lines.⁶⁻⁹

Consistent with this idea is that forced expression of p27^{KIP1} blocks cell cycle progression during the G₁ phase, whereas targeted p27^{KIP1} mRNA antisense vectors increase the fraction of cells in the S phase. Previous studies showed that p27^{KIP1} mRNA does not fluctuate during the cell cycle, implying the existence of posttranslational machinery that control animals at the p27^{KIP1} expression levels.¹⁰ The ubiquitin-mediated proteasome pathway was suggested to be involved in p27^{KIP1} degradation in mammals.¹¹ The ubiquitin-mediated proteasome pathway is emerging as a major and universal mechanism that regulates selective and time-controlled elimination of short-lived key regulatory proteins—for example, CKIs¹² or IκB,¹³ which was suggested to be involved in the differentiation of corneal epithelial cells in our recent articles.^{14,15}

The ubiquitin-mediated pathway of protein degradation comprises two discrete steps: the covalent attachment of multiple ubiquitin molecules to the protein substrate and the degradation of the polyubiquitylated protein by the 26S proteasome complex. Skp1-cullin-F-box protein (SCF) complex is one of the major classes of ubiquitin ligase and determines the specificity in protein ubiquitylation. Skp2 is an F-box protein that is a component of the SCF complex.¹⁶ Recently, it was reported that p27^{KIP1} is specifically recognized by Skp2 and that this step is a rate-limiting component of the machinery that ubiquitinates and degrades p27^{KIP1}.^{17,18} In the current study, the expression of p27^{KIP1} was examined in the normal and epithelial-scraped corneas. In addition, p27^{KIP1}^{-/-}, Skp2^{-/-}, and Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice were examined.

MATERIALS AND METHODS

Animals and Tissues

Development of the p27^{KIP1} Knockout, Skp2 Knockout, and p27^{KIP1}/Skp2 Double-Knockout Mice. C57Bl6 mice (9-12 weeks old) were obtained from Hokudo Corp., Sapporo, Japan. Skp2^{-/-} mice, p27^{KIP1}^{-/-} mice, and littermate controls (14 weeks old) were obtained as previously reported.^{19,20} Briefly, cloned genomic DNA corresponding to the p27^{KIP1} and Skp2 locus was isolated from a 129/Sv mouse genomic library. For the p27^{KIP1}^{-/-} mice, the targeting vector was constructed by replacing a 2.5-kb *SmaI-SmaI* fragment containing the entire p27^{KIP1} with a PGK-neo-poly(A) cassette. For the Skp2^{-/-} mice, the targeting vector was constructed by replacing a

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Submitted for publication April 11, 2001; revised October 2, 2001; accepted October 18, 2001.

Commercial relationships policy: N.

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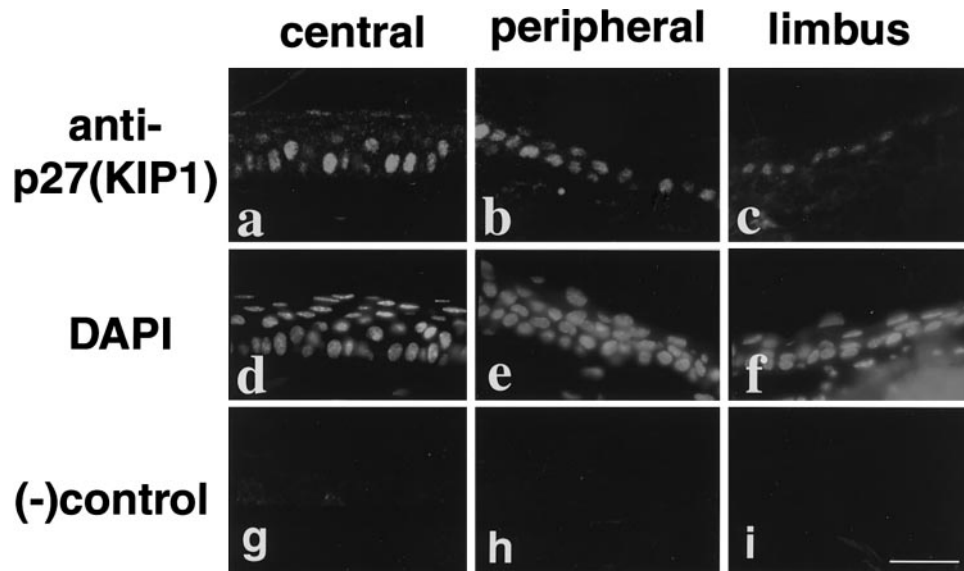


FIGURE 1. Immunodetection of p27^{KIP1} (a, b, c), DAPI staining (d, e, f), and the negative control to p27^{KIP1} staining (g, h, i) in the central (a, d, g) and peripheral (b, e, h) region of the cornea and in the limbus (c, f, i) of the C57Bl6 mouse. p27^{KIP1} was expressed in the nuclei of the basal cells in the central (a) and peripheral (b) region of the cornea and in the limbus (c). For a negative control to p27^{KIP1} staining, the serial sections without incubation of primary antibody were incubated with the FITC-conjugated goat anti-mouse IgG. Magnification, $\times 60$.

12-kb *SmaI-SacII* fragment containing exons 1 to 4 with a PGK-neo-poly(A) cassette. The maintenance, transfection, and selection of embryonic stem (ES) cells were performed as described.¹⁹ The mutant ES cells were microinjected in C57BL/6, and the resultant male chimeras were mated with female C57BL/6 mice. Double-heterozygote (*Skp2*^{+/-}/*p27*^{KIP1}^{+/-}) mice were interbred to generate mice deficient in both *Skp2* and *p27*^{KIP1}.

Cornea Wound Model. Epithelial scraping (1 mm diameter) was performed on the right-eye corneas, as previously described.^{21,22} Briefly, an epithelial wound was created by demarcating an area on the cornea with a 1-mm trephine and removing the epithelium within the circle with a small scalpel, leaving an intact basement membrane.²³ After the injury, ofloxacin ointment was applied to the eye to avoid an infection. The eyes treated in vivo after scrape-wounding did not have any sign of infection when observed by a dissection microscope.

The injured corneas were obtained at 6, 24, and 36 hours after the injury and fixed immediately with 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5) for 2 hours and processed for paraffin section. For bromodeoxyuridine (BrdU) labeling, the mice were injected with BrdU peritoneally at a dosage of 30 mg/kg body weight 30 minutes before sampling. The animal experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunocytochemistry

The slides were dewaxed, rehydrated, and rinsed in phosphate-buffered saline (PBS) twice and incubated with normal goat serum and then with the p27^{KIP1} antibody (dilution, 1:1000; Transduction Laboratories, Lexington, KY), which was generated from mouse p27^{KIP1}. Binding of the primary antisera was localized using FITC-conjugated goat anti-mouse IgG (dilution, 1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For the negative control of the p27^{KIP1} staining, the serial sections without incubation of primary antibody were incubated with the FITC-conjugated goat anti-mouse IgG. Nuclei were then stained by PBS containing 4'-6-diamidino-2-phenylindole (DAPI; 10⁻⁴ μ g/mL) for 5 minutes. Anti-BrdU staining was performed as described by Yoshiki et al.²⁴ Briefly, the sections were immersed in pepsin solution (0.4 mg/mL) in 0.1 N HCl at 30°C for 1 minute and

then in 2 N HCl at 40°C for 1 hour. After a wash in PBS, the slides were incubated with normal goat serum and then with the anti-BrdU antibody (dilution, 1:1000; Becton Dickinson, San Jose, CA). The slides were examined by laser scanning confocal microscopy (MRC-1024; Bio-Rad, Richmond, CA; and LSM 510; Carl Zeiss, Oberkochen, Germany). The BrdU-positive cells of the cornea were counted in sections from six each of the *Skp2*^{+/+}, *Skp2*^{-/-}, and *Skp2*^{-/-}/*p27*^{KIP1}^{-/-} double-knockout mice 24 hours after the injury, and significance was analyzed by the Aspin Welch *t*-test.

Assessment of Wound Area in the *Skp2*^{+/+} and the *Skp2*^{-/-} Mice

In *Skp2*^{+/+} and *Skp2*^{-/-} mice ($n = 6$ /group), the corneal epithelium was removed as previously described. To assess the wound area, fluorescein (Sigma Chemical Co., St. Louis, MO) diluted 2% in PBS was instilled onto the cornea. The wound area was determined from non-wound tissue by observation using a blue filter and monitored by corneal photography every 6 hours from the time the lesion was induced. The wound areas were analyzed on a computer (Macintosh; Apple Computer, Cupertino, CA), using the public domain NIH Image program (developed at the National Institutes of Health, Bethesda, MD, and available on the at <http://rsb.info.nih.gov/nih-image/>).

RESULTS

p27^{KIP1} was expressed in the nuclei of the basal cells of the central and peripheral region of the cornea and the limbus of the C57Bl6 mouse (Fig. 1). Because wounding of central corneal epithelium stimulates cell proliferation,¹ epithelial-scraped corneas of the C57Bl6 mouse were examined. In the sections from epithelial-scraped corneas sampled at 6 hours after injury, there was no staining of the p27^{KIP1}, either in the leading edge of the healing epithelium or in the peripheral region of the cornea or the limbus (Figs. 2a-c). p27^{KIP1} immunoreactivity was not detected in the central, peripheral, and

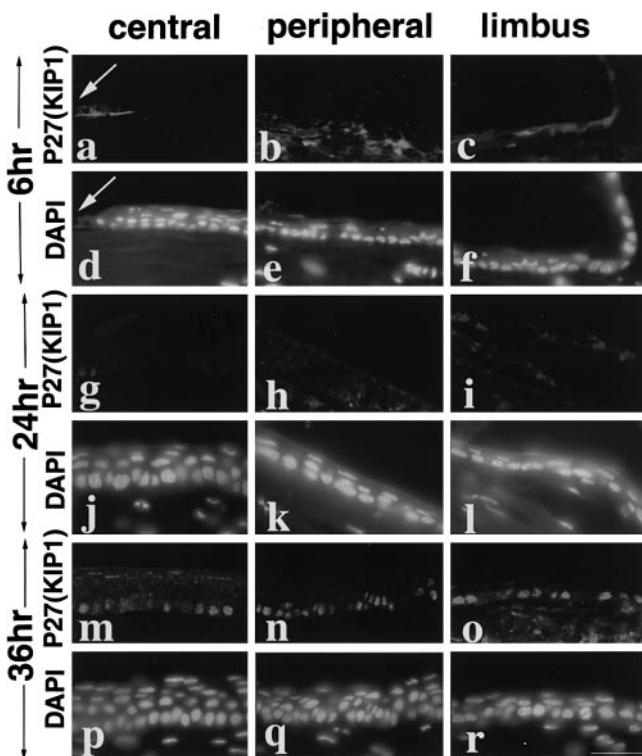


FIGURE 2. Immunodetection of p27^{KIP1} (a–c, g–i, m–o) and DAPI (d–f, j–l, p–r) staining in the cornea 6 (a–f), 24 (g–l), and 36 hours (m–r) after epithelial scraping of the cornea in the C57Bl6 mouse. (a, d) The leading edge (arrow) of the healing epithelium. Central (g, j, m, p) and peripheral (b, e, h, k, n, q) regions of the cornea. Limbus (c, f, i, l, o, r). Staining for p27^{KIP1} was not detected 6 and 24 hours after corneal scraping but was detected 36 hours after injury. Magnification, $\times 60$.

limbal region of the cornea after 24 hours (Figs. 2g–i) but was detected 36 hours after the injury (Figs. 2m–o).

Anti-BrdU staining of the scraped cornea of the C57Bl6 mouse at 6 hours after injury did not show any positive staining (Figs. 3a, 3c, 3e), whereas positive staining was observed at the basal cells of each area at 24 hours after injury (Figs. 3g, 3i, 3k). The number of BrdU-positive cells decreased at 36 hours after the injury (Figs. 3m, 3o, 3q).

To determine the proliferation of corneal epithelial cells in the absence of p27^{KIP1}, hematoxylin-eosin (HE) staining and

anti-BrdU staining were examined in the p27^{KIP1}^{−/−} mice (Fig. 4). There was no difference in the thickness and anti-BrdU staining of the corneal epithelium of uninjured p27^{KIP1}^{−/−} mice from that of control animals. Anti-BrdU staining of the scraped cornea of the p27^{KIP1}^{−/−} mouse at 6 hours after injury did not show any positive staining (Figs. 4o, 4s, 4w) and much positive staining was observed in the basal cells of each area at 24 hours after injury (Figs. 4p, 4t, 4x). Because it was reported that p27^{KIP1} was specifically recognized by Skp2 and this step was a rate-limiting component of the machinery that ubiquitinates and degrades p27^{KIP1},^{17,18} Skp2^{−/−} mice and Skp2^{−/−}/p27^{KIP1}^{−/−} double-knockout mice were examined. There was no obvious difference in the relative number of epithelial cell layers between uninjured Skp2^{+/+}, Skp2^{−/−}, and Skp2^{−/−}/p27^{KIP1}^{−/−} double-knockout mice (Fig. 5). The p27^{KIP1} staining in the Skp2^{−/−} was confined in the basal cells as in the Skp2^{+/+} mice.

Twenty-four hours after corneal scraping, Skp2^{+/+} mice had a three- to four-cell layer thickness in the epithelia of the cornea and limbus (Figs. 6a, 6d, 6g) whereas the Skp2^{−/−} mice had a one- to two-cell layer thickness (Figs. 6b, 6e, 6h). p27^{KIP1} staining was not detected 24 hours after epithelial scraping in the Skp2^{+/+} mice (Figs. 6j, 6m, 6p), whereas it was detected in the Skp2^{−/−} mice (Figs. 6k, 6n, 6q). BrdU staining of the cornea of the Skp2^{−/−} mice was also examined to determine whether Skp2 is involved in proliferation of basal cells of the corneal epithelium. Six hours after the procedure, positive cells for anti-BrdU staining were not detected in Skp2^{+/+} or Skp2^{−/−} mice (data not shown). There were many cells positive for anti-BrdU staining 24 hours after epithelial scraping in the Skp2^{+/+} mice (Figs. 6s, 6v, 6y), whereas few positive cells were detected in the Skp2^{−/−} mice (Figs. 6t, 6w, 6z). To examine whether the sustained expression of p27^{KIP1} is involved in the decrease in cell proliferation in the epithelium of Skp2^{−/−} cornea, Skp2^{−/−}/p27^{KIP1}^{−/−} double-knockout mice were examined. Twenty-four hours after epithelial scraping in Skp2^{−/−}/p27^{KIP1}^{−/−} double-knockout mice, epithelium of the cornea and limbus had a three- to four-cell layer thickness (Figs. 6c, 6f, 6i) and had many cells positive for anti-BrdU staining (Figs. 6u, 6x, 6#). The number of BrdU-positive cells in the cornea of the Skp2^{+/+} mice was significantly different from that in the Skp2^{−/−} mice ($P < 0.0001$) but not from that in the Skp2^{−/−}/p27^{KIP1}^{−/−} double-knockout mice (Fig. 7).

Thirty-six hours after the lesion in the Skp2^{−/−} mice, the number of p27^{KIP1}-positive cells was decreased, and there were many BrdU-positive cells in the corneal epithelium (Fig.

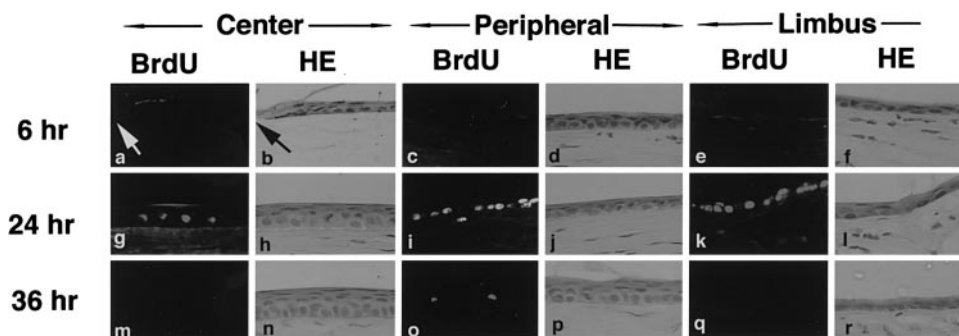


FIGURE 3. Anti-BrdU staining (a, c, e, g, i, k, m, o, q) and HE staining (b, d, f, h, j, l, n, p, r) in the cornea 6 (a–f), 24 (g–l), and 36 (m–r) hours after epithelial scraping of the cornea in the C57Bl6 mouse. (a, b) The leading edge (arrow) of the healing epithelium. Central (g, h, m, n) and peripheral (c, d, i, j, o, p) regions of the cornea. Limbus (e, f, k, l, q, r). Labeled nuclei were not detected after 6 hours but were detected 24 hours after the injury in all areas. The number of BrdU-positive cells declined at 36 hours after the injury (m–r). Magnification, $\times 60$.

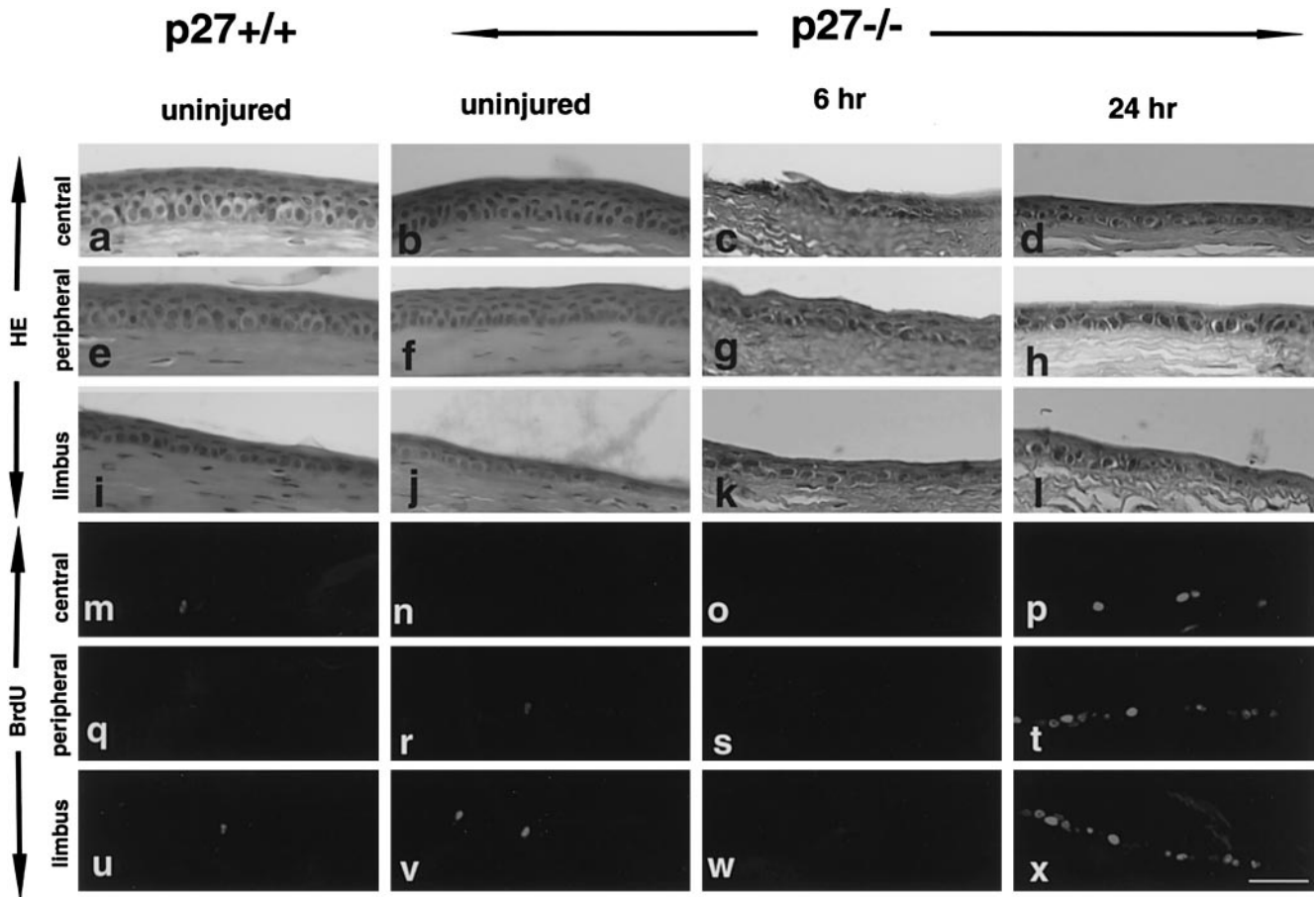


FIGURE 4. HE (a–l) and anti-BrdU (m–x) staining of central region (a, b, d, m, n, p), leading edge (c, o), and peripheral region (e–h, q–t) of the cornea and of the limbus (i–l, u–x) of the uninjured 14-week-old p27^{KIP1+/+} (a, e, i, m, q, u) and p27^{KIP1-/-} (b, f, j, n, r, v) mice and of the p27^{KIP1-/-} mice 6 hours (c, g, k, o, s, w) and 24 hours (d, h, l, p, t, x) after epithelial scraping. There was no difference in the thickness and anti-BrdU staining of the corneal epithelium in uninjured p27^{KIP1-/-} mice from that of control animals. Anti-BrdU staining of the scraped cornea of the p27^{KIP1-/-} mouse at 6 hours after injury did not show any positive staining (o, s, w) and much positive staining was observed in the basal cells of each area at 24 hours after injury (p, t, x). Bar, 40 μ m; magnification, \times 60.

8). The wound zone in the Skp2 null mouse was not significantly different from the wild-type mouse at each time point tested ($P > 0.05$; analyzed by the Breslow-Gegan Wilcoxon test) and was entirely covered 24 hours after the scraping (Fig. 9).

DISCUSSION

Degradation of the mammalian CDK inhibitor p27^{KIP1} is necessary for the cellular transition from quiescence to the proliferative state.¹⁹ In this study, the expression of p27^{KIP1} was

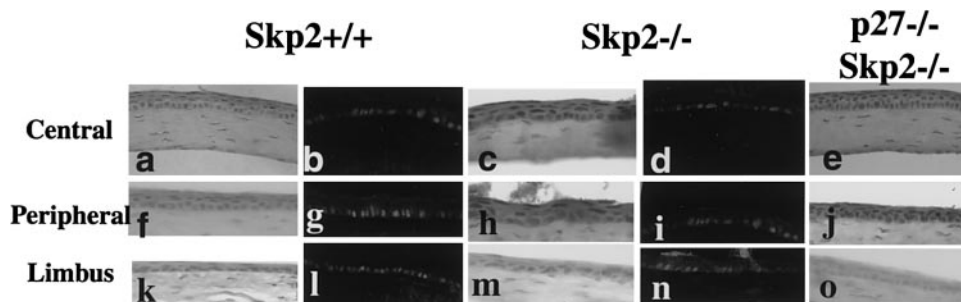


FIGURE 5. HE staining (a, c, e, f, h, j, k, m, o) and the immunodetection of p27^{KIP1} (b, d, g, i, l, n) in the central (a–e) and peripheral (f–j) cornea and in the limbus (k–o) of 14-week-old Skp2^{+/+} (a, b, f, g, k, l), Skp2^{-/-} (c, d, h, i, m, n), and Skp2^{-/-}/p27^{KIP1-/-} double-knockout mice (e, j, o). There was no obvious difference in the relative number of epithelial cell layers between Skp2^{+/+}, Skp2^{-/-}, and Skp2^{-/-}/p27^{KIP1-/-} double-knockout mice. p27^{KIP1} was expressed in the nuclei of the basal cells in the central (d) and peripheral (i) regions of the cornea and in the limbus (n) of the Skp2^{-/-} and Skp2^{+/+} mice (b, g, l). Bar, 20 μ m; magnification, \times 40.

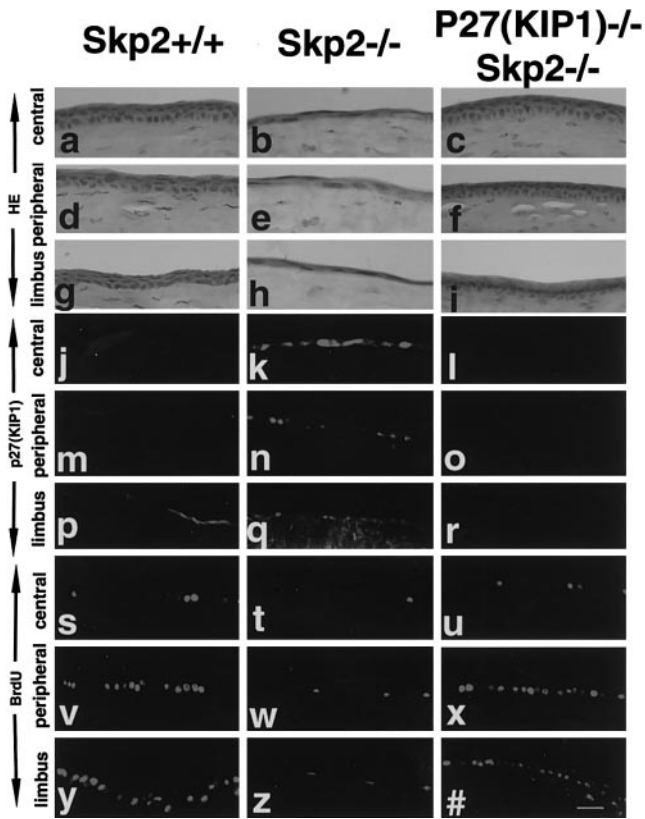


FIGURE 6. HE staining (a–i), the immunodetection of p27^{KIP1} (j–r), and anti-BrdU staining (s–#) of the central cornea (a–c, j–l, s–u), peripheral cornea (d–f, m–o, v–x), and limbus (g–i, p–r, y–#) 24 hours after corneal scraping in the Skp2^{+/+} (a, d, g, j, m, p, s, v, y), Skp2^{-/-} (b, e, h, k, n, q, t, w, z), and Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout (c, f, i, l, o, r, u, x, #) mice. The epithelium in the cornea and limbus of the Skp2^{+/+} mice was of three- to four-cell layer thickness (a, d, g), whereas that of the Skp2^{-/-} mice was of one- to two-layer thickness (b, e, h). p27^{KIP1} staining was not detected in the corneal epithelium of the Skp2^{+/+} mice (j, m, p), whereas it was detected in that of the Skp2^{-/-} mice (k, n, q). There were many cells positive for anti-BrdU staining in the peripheral cornea (v) and limbus (y) 24 hours after epithelial scraping in the Skp2^{+/+} mice, whereas few positive cells were detected in the Skp2^{-/-} mice (w, z). Twenty-four hours after epithelial scraping in the Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice, the peripheral cornea and limbus were three to four cell layers thick (c, f, i) and had many cells positive for anti-BrdU staining (x, #). Bar, 20 μm; magnification, ×60.

examined in the mouse cornea. p27^{KIP1} was expressed in basal cells of the central and peripheral region of the cornea and limbus. In the sections from epithelial-scraped cornea sampled at 6 and 24 hours after injury, there was no staining with p27^{KIP1} (Fig. 2). Cells in the S phase of DNA synthesis were not detected at 6 hours after injury, but were detected at 24 hours after injury (Fig. 3). These results suggest that disappearance of p27^{KIP1} was well correlated with cell proliferation in the corneal epithelium.

To determine whether the absence of p27^{KIP1} was sufficient for the proliferation of corneal epithelial cells, p27^{KIP1}^{-/-} mice were examined (Fig. 4). There was no obvious difference in the thickness and anti-BrdU staining in the corneal epithelium between p27^{KIP1}^{-/-} mice and control animals. The absence of ectopic or inappropriate proliferation in nonlesioned cells in the cornea of p27^{KIP1}^{-/-} mice suggests that absence of p27^{KIP1} is not sufficient for proliferation of corneal epithelial cells.

Because p27^{KIP1} was specifically recognized by Skp2, and this step was a rate-limiting component of the machinery that ubiquitinates and degrades p27^{KIP1},^{17,18,20} Skp2^{-/-} mice were examined (Fig. 5, 6). p27^{KIP1} staining was not detected in the epithelium 24 hours after epithelial scraping in the Skp2^{+/+} mice, whereas it was detected in the Skp2^{-/-} mice. Few cells were in the S phase of DNA synthesis 24 hours after injury in the epithelium of the Skp2^{-/-} mice. These results suggest that Skp2 is involved in the degradation of p27^{KIP1} and regulation of cell proliferation in the corneal epithelium.

There was a possibility that a decrease in cell proliferation in the Skp2^{-/-} cornea was not caused by the defect in the degradation of p27^{KIP1}, but by the degradation of cyclin E or other protein, because Skp2 is also involved in the degradation of cyclin E.²⁰ To exclude this possibility, Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice were examined (Fig. 5, 6). Twenty-four hours after the epithelial scraping in Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice, the epithelia of the cornea and limbus were three to four cell layers in thickness and had many anti-BrdU-positive cells. These results suggest that the decrease in cell proliferation in the Skp2^{-/-} cornea was caused by sustained expression of p27^{KIP1}.

During the early stage of scrape injury, epithelial cell migration was observed. After the covering of the bare surface, epithelial proliferation started in some zones behind the leading edge.^{1,21} Because the rate of covering the scrape in the Skp2^{-/-} mice was almost the same as that in the Skp2^{+/+} mice (Fig. 9), we hypothesized that the covering of the scrape in our experiments is solely dependent on migration and adhesion.

In this study, suprabasal cells in the corneal epithelium were negative for p27^{KIP1}. p27^{KIP1} was upregulated in the retinal neurons as they exited the cell cycle and was downregulated after the terminal mitosis.²⁵ Expression of p27^{KIP1} in the postmitotic suprabasal cells may be also downregulated after terminal mitosis.

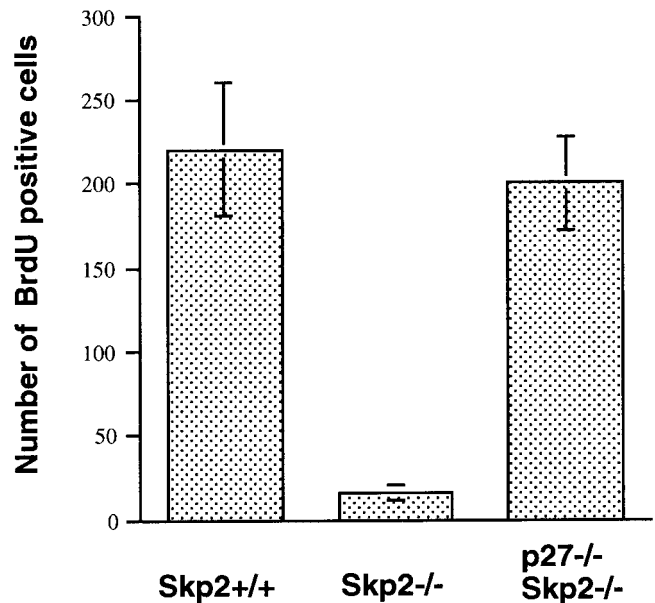


FIGURE 7. Number of BrdU-positive cells in the cornea of Skp2^{+/+}, Skp2^{-/-}, and Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice 24 hours after epithelial scraping. Bars represent the mean ± SD of BrdU-positive cells in the cornea. The number of BrdU-positive cells in the cornea of the Skp2^{+/+} mice was significantly different from the Skp2^{-/-} mice ($P < 0.0001$) but not from Skp2^{-/-}/p27^{KIP1}^{-/-} double-knockout mice.

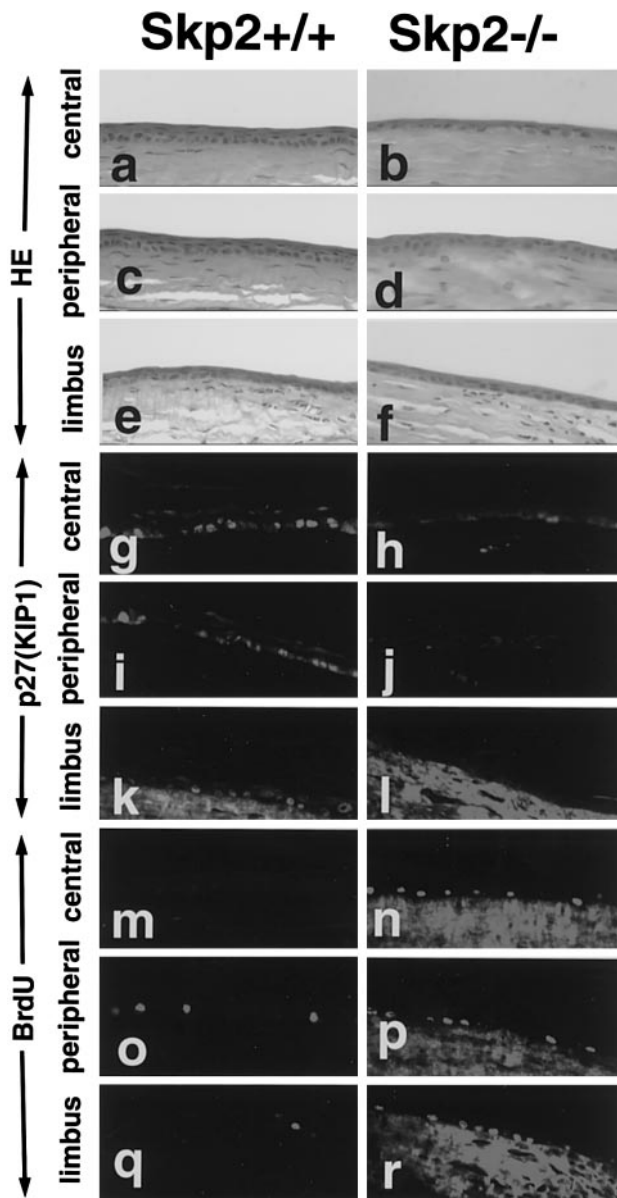


FIGURE 8. HE staining (a–f), the immunodetection of p27^{KIP1} (g–l), and anti-BrdU staining (m–r) of the central (a, b, g, h, m, n) and peripheral cornea (c, d, i, j, o, p) and of the limbus (e, f, k, l, q, r) 36 hours after corneal scraping in the Skp2^{+/+} (a, c, e, g, i, k, m, o, q) and Skp2^{-/-} (b, d, f, h, j, l, n, p, r) mice. The epithelium of the Skp2^{-/-} mice was thinner than that of the Skp2^{+/+} mice. There were many cells positive for p27^{KIP1} in the epithelium of the Skp2^{+/+} mice (g, i, k) but not in that of Skp2^{-/-} mice (h, j, l). There were few positive cells for anti-BrdU staining in the epithelium of the Skp2^{+/+} mice, whereas many positive cells were detected in the Skp2^{-/-} mice (n, p, r). Magnification, $\times 40$.

Thirty-six hours after the lesion, the number of p27^{KIP1}-positive cells in the corneal epithelium of the Skp2^{-/-} mice was decreased, whereas it was increased in the epithelium of the Skp2^{+/+} mice (Fig. 8). This delay in disappearance of p27^{KIP1} was well correlated with the delay in BrdU incorporation in the Skp2^{-/-} mice. The decrease in the number of p27^{KIP1}-positive cells in the corneal epithelium of the Skp2^{-/-} mice may be related to ubiquitin-independent processing during progression from the G₁ to the S phase²⁶ or to a ubiquitin-dependent, but Skp2-independent, mechanism.

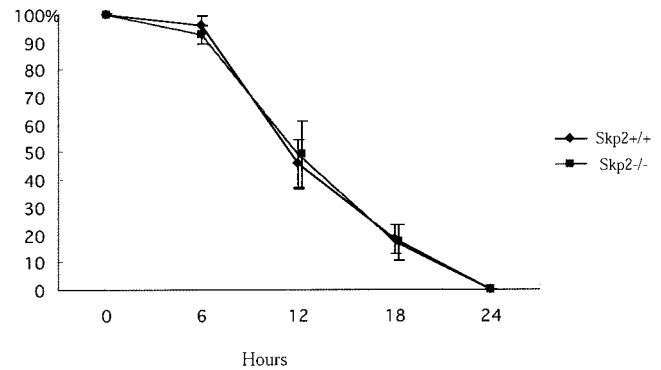


FIGURE 9. The wound area after corneal scraping in the Skp2^{+/+} and the Skp2^{-/-} mice. The wound area relative to that measured just after the procedure is expressed as a percentage. Each value represents the mean \pm SD of six animals per time point in each group.

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