Sonic Hedgehog Expression and Role in Healing Corneal Epithelium

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Purpose. To examine the expression pattern and roles of Sonic hedgehog (Shh) in healing corneal epithelium.

Methods. Immunofluorescent staining and Western blot analysis were used to detect Shh, patched 1 (Ptc 1) receptors, and Gli transcription factors in corneal epithelium of Wistar rats (n = 44) at various intervals after an epithelial defect. Effects of exogenous Shh on cell proliferation and cyclin D1 expression were determined in healing corneal epithelium of organ-cultured mouse eyes.

Results. Uninjured rat corneal epithelium was not labeled by anti-Shh antibody, but weakly positive for Ptc 1. Basal cells of limbal and conjunctival epithelia were labeled by antibodies against Shh and Ptc 1. Shh protein was transiently upregulated in limbal epithelium in 2 hours and was also transiently expressed in the migrating corneal epithelium with its peak at 12 hours postdebridement. Such upregulation of Shh expression was associated with a transient nuclear translocation of Gli-3 without lifting the suppression of cell proliferation in migrating epithelium postdebridement in vivo. However, an addition of Shh protein to culture medium resulted in nuclear accumulation of cyclin D1 and marked acceleration of epithelial cell proliferation in migrating corneal epithelium of an organ-cultured mouse eye.

Conclusions. Corneal epithelial debridement causes a transient upregulation of Shh expression and activation of Shh/Gli-3 signaling cascade in healing corneal and limbal epithelia. Although exogenous Shh promotes epithelial cell proliferation in corneal organ culture, its expression in migrating epithelium in vivo does not counteract the suppression of cell proliferation at the early healing phase of epithelium debridement. (Invest Ophthalmol Vis Sci. 2004;45:2577–2585) DOI:10.1167/iovs.04-00011

Hedgehog is a family of secreted proteins that serve as morphogens during development.1,2 In mammals three hedgehog homologues have been identified: Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog.1,2 Shh is involved in the left–right asymmetry decision, anterior–posterior axis decision in limb pattern determination, and hair follicle formation during embryonic development.1,2 Shh modulates proliferation of dental epithelium, and proliferation and differentiation of epidermal cells of the hair follicle and the gastrointestinal tract epithelium.3–5 Shh also contributes to the specification of dorso-ventral patterning in the spinal cord and the proliferation and differentiation of neural precursors.6,7 Mouse Shh mRNA encodes a protein of 457 amino acids, which is post-translationally modified by an autocatalytic reaction to yield a bioactive 19 kDa N-terminal domain and a 27 kDa C-terminal domain that is involved in intramolecular processing. Binding of Shh to transmembrane receptors of the Patched (Ptc) family (e.g., Ptc 1) activates Smoothened (Smo), another transmembrane protein that is suppressed in the absence of Shh signal.1,2 Gli is a family of transcription factors consisting of three members (Gli-1, -2, and -3). Gli is activated and translocated to nuclei on activation of Ptc 1/Smo signaling pathway by Shh, and mediates Shh-dependent gene expression.8

The Shh/Ptc 1/Smo pathway reportedly directly regulates the cell cycle.9,10 Although the mechanism of Shh regulation is not well understood, it has been suggested that Shh promotes cell proliferation via upregulating cyclin D1.10 For example, Shh promotes proliferation of human keratinocytes in vitro and in situ by counteracting p21Cip1, a cyclin-dependent kinase inhibitor involved in physiological growth arrest.11,12 Such an accelerating effect on epithelial cell proliferation may be involved in the development of skin neoplasm. In fact, mutations in the components involved in Shh/Ptc pathway have been detected in basal cell carcinoma of the skin and medulloblastoma.13–18 Overexpression or activating mutation of any components of Shh cascade (e.g., Gli or Smo) are sufficient to promote the formation of basal cell carcinoma in mice.13–18

The regulatory role of Shh in cell proliferation during development and neoplasm formation suggests that this molecule may also participate in modulation of tissue repair. Indeed, the Shh/Ptc/Gli cascade underlies the tissue repair process of airway epithelium and epidermis.19–20 Nuclear translocation of Gli-1 associated with Shh upregulation was detected in cells of airway epithelium at day 1 of healing after an epithelial injury caused by naphthalene inhalation.20 Epidermal keratinocytes of adult rat also upregulate Shh on full-thickness incision (Kishi K et al., personal communication, 2003).

These observations led to the hypothesis that Shh may also play a role in corneal epithelial wound healing.21–24 Immunohistochemistry and Western blot analysis of Shh and Ptc 1 in healing rat corneal epithelium after debridement showed that Shh was upregulated in migrating corneal epithelium. To further examine the signaling of Shh in migrating corneal epithelial cells, Gli members (Gli-1 and Gli-3) were immunohistochemically located. Ki67 expression was determined to elucidate the cell proliferation of healing rat corneal epithelium in vivo. To correlate cell proliferation and Shh, the effect of exogenous Shh on cyclin D1 expression and epithelial cell proliferation was examined using bromodeoxyuridine (BrdU) incorporation and expression of proliferating cell nuclear antigen (PCNA), in organ-cultured mouse corneas.
MATERIALS AND METHODS
Corneal Epithelial Wound Healing in Rats
Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approvals of the Institutional Animal Care and Use Committees of Wakayama Medical University. Wistar rats (n = 24) were generally anesthetized by ether inhalation and i.p. pentobarbital sodium (6.5 mg/100 gram body weight), as previously reported. Central corneal epithelium 2.4 mm in diameter in one eye was debrided with a dull scalpel after administration of oxybuprocaine eyedrop (Santen, Osaka, Japan). The other eye served as control. An antibiotic ointment was applied to both eyes. After healing intervals of 1, 2, 6, 12, and 24 hours, the experimental animals were killed by inhalation of ether and an overdose of pentobarbital sodium. Eyes were enucleated and embedded in OCT compound. Cryosections (7 μm thick) were fixed in cold acetone for 5 minutes and processed for immunohistochemistry as described below.

Western Blot Analysis of Shh in Healing Corneal Epithelium
A central corneal epithelial defect was made as described above in the right eyes of Wistar rats (n = 20). The left eye served as control. After 12 hours, the animals were killed. Corneal epithelium or conjunctival epithelium (including limbal epithelium) of uninjured eyes and corneal epithelium at 12 hours postdebridement were collected in phosphate-buffered saline (PBS) and lysed in lysis buffer (CellLytic MT; Sigma, St. Louis, MO). Approximately 200 μg protein/10 μL was subjected to 5–20% SDS-polyacrylamide gel electrophoresis. Protein was transferred to a PVDF membrane (Immobilon-P; Millipore, Bedford, MA), and treated with PBS containing 5% dried milk for 30 minutes. The membrane was then reacted with anti-Shh antibody (0.2 μg/mL) in PBS supplemented with 1% dried milk at 4°C overnight. After washing in PBS and 4 hour-treatment with a peroxidase-conjugated secondary anti-goat immunoglobulin antibody diluted in PBS containing 1% dried milk, the immunoreactive protein was visualized by using an enhanced chemiluminescence kit (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK).

Effect of Mouse Recombinant Shh on Healing Corneal Epithelium
Adult male C57/BL6 mice were generally anesthetized by i.p. pentobarbital sodium. Central corneal epithelium 2 mm in diameter was debrided and the animals were subsequently killed by CO2 asphyxia and cervical dislocation without being awakened. An enucleated eye was placed in a well of a 24-well culture plate containing 1 mL of serum-free Dulbecco’s minimum essential medium supplemented with 2.5 or 5.0 μg/mL of mouse recombinant Shh or bovine serum albumin (Sigma). Four eyes were prepared for each culture condition. After 9 and 20 hours of culture at 37°C, the globes were labeled with BrdU for 2 hours as previously reported. At the end of culture, each globe was observed after regular light microscopy. PCNA immunostaining was performed as described above. Negative control stainings were performed by using species-matched nonimmune host immunoglobulins at the same concentration as each primary antibody.

Organ-cultured specimens were processed for immunohistochemistry for BrdU, PCNA, and cyclin D1 by using monoclonal anti-BrdU antibody (1:10 dilution in PBS; Roche-Boehringer-Mannheim, Indianapolis, IN), monoclonal anti-PCNA antibody (1:100 dilution in PBS; Santa Cruz Biotechnology) and rabbit monoclonal anti-cyclin D1 antibody (1:100 dilution in PBS; Cell Signaling Technology, Beverly, MA) as described above. For BrdU immunohistochemistry, the tissue sections were treated with 2 N HCl for 2 hours at 37°C and then processed for blocking and primary antibody reaction as previously described. After a secondary peroxidase-conjugated antibody reaction and washing in PBS, the antibody complex was visualized with 3,3’-diaminobenzidine. After nuclear counterstaining with methylgreen, the specimens were observed under regular light microscopy. PCNA immunofluorescent staining was performed as described above. Negative control was done with species-matched host nonimmune immunoglobulin at the same concentration of each primary antibody.

RESULTS
Shh Expression in Epithelium of Healing, Injured, and Uninjured Rat Cornea
Immunofluorescence staining was performed to examine Shh expression patterns in normal and injured ocular surface epithelia. In normal, uninjured rat eye, Shh protein was detected in basal epithelial cells of limbus and bulbar and palpebral conjunctiva, but not in corneal epithelium (Figs. 1 and 2). Immunofluorescence signals found in basal cells of conjunctival epithelium were abruptly discontinued at the epidermis juxtaposed to palpebral conjunctiva (Figs. 1A, 1C–1E). Basal cells of hair follicles and sebaceous glands were also positively stained (Figs. 1A, 1B).

**FIGURE 1.** Immunofluorescent staining for Sonic hedgehog (Shh) in ocular epithelia. Shh is immunohistochemically detected in basal cells of palpebral conjunctival epithelium and sebaceous gland cell (arrow, Panel A), whereas it is not observed in eyelid epidermis. Panel B shows Shh expression hair follicles (arrows). Panels C, D, and E indicate a continuous expression in basal cells of the palpebral, forni-cal, and bulbar conjunctival epithelia. Bar, 100 μm.
A Western blot analysis further confirmed the transient expression of Shh in migrating corneal epithelium as revealed by immunofluorescence staining. The result showed a protein of molecular weight around 46 kDa immunoreactive to anti-Shh antibody in healing corneal epithelium and uninjured conjunctival epithelium (including limbal epithelium), but not seen in uninjured corneal epithelium (Fig. 4).

**Ptc 1, Gli-1, and Gli-3 Expression in Healing, Injured, and Uninjured Rat Corneal Epithelium**

Ptc 1 was immunohistochemically detected weakly, but positively, in corneal epithelium and keratocytes, regardless the presence or absence of an epithelial defect, and its immunofluorescent intensity did not alter during healing interval (data not shown). Basal cells of limbal and conjunctival epithelia also expressed Ptc 1 (data not shown).

Gli family members are transcription factors involved in Shh/Ptc1/Smo signaling. To examine if Shh protein upregulation in migrating corneal epithelium triggers Shh/Ptc1/Smo signaling, Gli proteins were localized by immunofluorescent staining. Gli-1 was detected in corneal epithelium throughout the healing interval without any noticeable alteration of intracellular localization in epithelium (data not shown), while Gli-3
translocated transiently to nuclei of basal epithelial cells in migrating epithelium (Fig. 5). In normal uninjured corneal epithelium and that at 1 hour postinjury (not shown), Gli-3 was detected in the cytoplasm and cell–cell border area with a faint nuclear staining in some of the basal cells (Fig. 5aA). Immunoreactivity in the cytoplasm seemed more marked in the migrating epithelium at 2 hours postdebridement compared with uninjured epithelium (Fig. 5aC). At 6 hours (Fig. 5aE) and 12 hours (data not shown) postinjury, Gli-3 protein was detected in the nuclei of migrating epithelium. At 12 hours (data not shown) and 24 hours (Fig. 5aG), Gli-3 immunofluorescence in the epithelium was reduced. Upregulation of Shh in limbal epithelium was accompanied by the activation and nuclear translocation of Gli-3. Cell nuclei of an uninjured limbal epithelium were negative for Gli-3 (Fig. 5aB), whereas nuclear translocation of Gli-3 was detected at 2 hours postdebridement (arrows, Fig. 5aD). At 6 hours, only a minority of limbal basal cells showed a nuclear Gli-3 immunoreactivity (arrow, Fig. 5aF), and at 12 hours (not illustrated) and 24 hours (Fig. 5aH) such nuclei no longer showed Gli-3 immunoreactivity.

**FIGURE 4.** Western blot analysis of Shh expression in migrating rat corneal epithelium. Shh was not detected in uninjured corneal epithelium (lane 1), whereas it was observed in healing corneal epithelium at 12 hours postinjury (lane 2), as well as in uninjured conjunctival epithelium including limbal epithelium (lane 3).

**FIGURE 5.** Activation of Gli-3 signaling and proliferation of epithelial cells in healing epithelium postdebridement in vivo. (a) Immunofluorescent detection of Gli-3 in healing rat corneal and limbal epithelium. Gli-3 protein is observed in the cell cytoplasm of normal, uninjured, corneal (aA) and limbal (aB) epithelia. At 2 hours postdebridement, nuclei of healing corneal epithelium remain negative for Gli-3 (aC), whereas nuclear translocation of Gli-3 was detected in limbal epithelium (arrows, aD). Gli-3 protein is then observed in many cell nuclei of migrating epithelium at 6 hours (arrows, aE) and 12 hours (data not shown) postinjury. At these timepoints only a few limbal basal cells show a nuclear Gli-3 immunoreactivity (arrow, aF). At 24 hours postinjury, Gli-3 immunofluorescent staining is no longer observed in both corneal (aG) and limbal (aH) epithelia. Inset in (aD): a high magnification picture of nuclear localization of Gli-3. Bar, 100 μm. (b) Distribution of Ki67-positive cells in corneal epithelium at intervals of healing postdebridement. Many Ki67-labeled cells are observed in the basal layer of uninjured corneal epithelium (bA, bB). The number of Ki67-positive cells decreases in healing epithelia at 1 hour (not illustrated), 2 hours (bC, bD), and 6 hours (bE, bF) postdebridement. At 12 hours postinjury, the healing epithelium is still less proliferative, whereas many of the repopulated keratocytes are labeled with anti-Ki67 antibody (not illustrated). At 24 hours, nuclei of many cells of central epithelium resurfacing the defect are labeled with anti-Ki67 antibody (bG, bH). Immunofluorescent staining (bB, bD, bF, and bH: DAPI nuclear staining). (c) Histogram of Ki67-positive epithelial cells at each healing interval. Bar, 100 μm.
Sonic Hedgehog in Epithelial Healing

In Vivo Epithelial Cell Proliferation of Injured Rat Cornea

Shh modulates (accelerates in most cases) proliferation of epithelial cells in many tissues. Therefore the proliferation of healing rat corneal epithelium was examined by immunostaining of Ki67 antigen, the expression of which is correlated with BrdU incorporation.27 In uninjured corneal epithelium some basal cells were stained with the anti-Ki67 antibody. During healing of epithelial debridement, the number of Ki67-positive cells in migrating epithelium decreased. In detail, the number of Ki67-positive cells in the epithelium adjacent to the defect was reduced at 1 hour postinjury (not illustrated), compared with uninjured epithelium (Figs. 5bA and 5bB). Only a small number of labeled cells could be seen in the migrating epithelium at 2 hours (Figs. 5bC, 5bD, 5bE, 5bF, 6) and 12 hours (not illustrated) postdebridement. The healing epithelium restored cell proliferation activity at 24 hours of debridement (Figs. 5bG, 5bH). The result of counting Ki67-positive cell numbers is summarized in the graph in Figure 5c.

As for the limbal epithelium, no significant alteration in the numbers of Ki67-positive basal epithelial cells was detected during the healing intervals examined (data not shown), although Gli-3 translocated to nuclei.

Effect of Recombinant Mouse Shh Protein on Healing of Corneal Epithelium Debridement in Organ Culture of Mouse Eyes

Immunostaining with anti-Ki67 antibody revealed that the numbers of cells labeled by the antibody in vivo were not correlated with upregulated Shh protein expression and Gli-3 nuclear translocation in healing rat corneal epithelium. Shh is believed to accelerate the cell cycle in epithelial cell types. Thus, the role of Shh on cell proliferation in migrating corneal epithelium was analyzed in an organ-culture model of wound healing in mouse corneas.

Adding Shh protein (2.5 μg/mL) to the medium did not significantly affect the closure rate of the corneal epithelial defect (data not shown), compared with control specimens. Addition of 5.0 μg/mL Shh protein slightly, but significantly, accelerated defect closure rate at 22 hours (Fig. 6). Immunodetection of PCNA (Fig. 7) or BrdU (data not shown) showed a marked increase in the number of PCNA-positive cells in the entire epithelium at culture intervals of 11 hours (Figs. 7A–7D) and 22 hours (Figs. 7E–7H). The number of PCNA-labeled epithelial cells in a 200 μm length migrating epithelium was statistically significantly increased in the Shh-plus cultures in comparison to control cultures (Table 1).

Cyclin D1 Expression in Corneal Epithelium Organ-Cultured in the Presence of Shh

To further elucidate the mechanism of cell proliferation acceleration by Shh, the expression and intracellular location of cyclin D1 protein were examined in healing corneal epithelium of organ-cultured mouse eyes in the presence of 5.0 μg/mL recombinant Shh. Cytoplasm of migrating corneal epithelial cells at 11 hours of culture was faintly immunostained for cyclin D1 in control specimens (Fig. 8A), whereas nuclei of epithelial cells cultured in the presence of Shh were markedly labeled by the antibody (Fig. 8B). At 22 hours of culture, immunoreactivity for cyclin D1 was still present in cell cytoplasm in central (Fig. 8E) and peripheral (Fig. 9I) epithelia in control, Shh-minus, cultures. Nuclear staining was observed in many cells of central (Fig. 8F) and peripheral (Fig. 8J) epithelia in the presence of Shh at 22 hours. Taken together, these observations suggest that Shh added to culture medium promotes expression and nuclear translocation of cyclin D1 and cell proliferation in healing epithelium of ex vivo cultured mouse eyes.

DISCUSSION

The present study revealed for the first time that Shh protein is upregulated in migrating corneal epithelium after debridement, as well as constantly expressed in basal cells of uninjured
limbal and conjunctival epithelium. Corneal epithelium was labeled by antibody against Ptc 1, the Shh receptor, suggesting that Shh may modulate epithelial behavior in an autocrine/paracrine fashion. Further examination indicated that a transient nuclear translocation of Shh-specific transcription factors, Gli-3, but not Gli-1, occurred during the healing of injured corneal epithelium, suggesting that the binding of Shh to Ptc 1 receptor-activated Gli-3.

Shh protein reportedly promotes proliferation of epithelial cell types (e.g., epidermal keratinocytes) in cell culture.\(^{19,20}\) The mechanism by which Shh accelerates cell proliferation reportedly includes upregulation of cyclin D1 and counteraction of the inhibitory effect of p21\(^{CIP1}\). The present organ-culture experiments also showed that exogenous Shh protein promoted cell proliferation in healing mouse corneal epithelium, which was accompanied by an upregulation and nuclear translocation of cyclin D1. On the other hand, no significant increase of Ki67-positive cells was detected in either the migrating corneal epithelium or limbal epithelium during central epithelial healing in vivo in rats, although immunofluorescent staining showed an activation of Shh/Gli-3 signaling. Therefore, the transient upregulation of Shh and subsequent activation of Shh/Gli-3 signaling in vivo did not lift the suppression of cell proliferation in migrating epithelium at the early healing stage of central epithelium debridement, suggesting the presence of a mechanism counteracting Shh accelerating effects on cell proliferation. For example, Zieske et al. reported that migrating corneal epithelium upregulates TGF\(\beta\) receptors.\(^{28}\) TGF\(\beta\) suppresses cell proliferation of epithelial cell types by upregulating p15\(^{INK4B}\)\(^{32-35}\) and p21\(^{CIP1}\) mediated through Smad\(^{33-35}\) and/or non-Smad\(^{36-38}\) pathways. Migrating corneal epithelial cells lack nuclear Smads\(^3/4\)\(^{26}\) and loss of Smad3 does not affect cell proliferation of healing corneal epithelium after debridement in mice (Saika S, unpublished data, 2003), suggesting that the Smad signal might not be activated in migrating corneal epithelium. Therefore, it is likely that TGF\(\beta\) suppression of cell proliferation is mediated via non-Smad induction\(^{36-38}\) of p21\(^{CIP1}\) and activation of p38MAPK\(^{28}\) rather than by Smad cascades, although the mechanism that suppresses cell proliferation in migrating corneal epithelium is to be clarified. This suggestion is substantiated by the recent observation that inhibition of p38MAPK by specific inhibitors lifts the suppression of cell proliferation in healing corneal epithelium, while it abolishes epithelium migration. Shh is capable of upregulation of TGF\(\beta\), which suppresses epithelial cell proliferation, in nocular tissues (prostate and bone) during development.\(^{39,40}\)

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Immunofluorescent detection of proliferating cell nuclear antigen (PCNA) in mouse corneal organ-cultured for 11 or 22 hours with 5.0 mg/mL of mouse recombinant Sonic hedgehog (Shh) protein. (A) and (B) indicate each side of the migrating epithelium in one cornea in a control, bovine serum albumin-plus, culture, shown in (C) and (D) in a Shh-plus culture at 11 hour culture interval. (B') and (C') are high magnification pictures of the boxed areas seen in (B) and (C). More PCNA-expressing epithelial cells are observed in a cornea treated with Shh protein compared with that in the control culture. (E) and (F) indicate each side of the migrating epithelium in one cornea in a control, bovine serum albumin-plus, culture, and (G) and (H) show that in a Shh-plus culture at 22 hour culture interval. Similarly to the specimens of 11 hour culture, PCNA-positive cells are more frequently observed in a Shh-treated specimen compared with a control specimen. Bar: (A, B, C, and D) 600 \(\mu m\); (B’, C’) 100 \(\mu m\).
Although these reports suggest that TGFβ signaling is required for eliciting the effects of Shh, it has not been clarified whether a similar mechanism underlies wound healing in the corneal epithelium. Further study is needed to characterize the signaling pathways modulating cell proliferation in migrating corneal epithelium during healing of debridement.

The present study also showed an upregulation of Shh/Gli-3 signaling in limbal epithelium, whereas the central epithelial defect used did not induce an increment of cell proliferation in limbal epithelium. Activation of Gli-3 signaling was observed with an increment of immunofluorescent staining for Shh in limbal epithelium as early as at 2 hours postdebridement, when such signaling was still not activated in migrating corneal epithelium. The roles of Shh upregulation and Gli-3 activation in limbal epithelium after corneal epithelial debridement remain to be defined.

Shh is also involved in cell differentiation in many organ systems: dental progenitor epithelium, or fundic gland in adult human gastrointestinal tract.2–5 Cell culture studies further show roles of Shh in types of cell differentiation. For example, capillary formation by cultured vascular endothelial cell is promoted by adding Shh to the medium.41 The present organ culture experiment showed no difference in the expression of keratin 12, a marker of cornea-type epithelial differentiation in healing corneal epithelium in the presence and absence of Shh (data not shown). The roles of Shh in modulation of intraepi-

| TABLE 1. The Number of PCNA-Labeled Epithelial Cells in 200 μm Length Migrating Epithelium |
|-----------------------------------------------|---------------|
| 11 hours | 22 hours |
| Control 6.5 ± 1.7 | 7.5 ± 1.7 |
| Shh 2.5 μg/ml 17.0 ± 6.3* | 15.3 ± 4.6 |
| Shh 5.0 μg/ml 45.5 ± 5.5** | 24.3 ± 6.9* |

Significant * P < 0.05 or ** P < 0.01 by unpaired t-test.
thelial differentiation from basal cells toward superficial cells remain to be investigated.

References


Figure 8. Expression of cyclin D1 in corneal epithelium organ-cultured in the presence and absence of 5.0 μg/ml of recombinant Sonic hedgehog (Shh) protein. (A, B, E, F, I, and J) indicate the expression of cyclin D1 by green fluorescence; (C, D, G, H, K, and L) show the localization of cell nuclei as stained by DAPI. Cytoplasm of migrating corneal epithelial cells at 11 hours of culture was faintly immunostained for cyclin D1 in control specimens (A), whereas nuclei of epithelial cells cultured in the presence of Shh were markedly labeled with the antibody (B). At 22 hours of culture, immunoreactivity for cyclin D1 was still present in cell cytoplasm in central (E) and peripheral (I) epithelia in control, Shh-null, cultures. However obvious nuclear staining was observed in many cells of Shh-treated central (F) and peripheral (J) epithelia at 22 hours. Bar, 10 μm.


