Developmentally Regulated Expression of Sp1 in the Mouse Cornea

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Purpose. To examine the expression of transcription factor Sp1 in the cornea of the mouse eye throughout developmental stages. The environmental effect of light on Sp1 expression was also assessed.

Methods. C57BL/6 mice were set up for timed mating. Embryos on embryonic day (E)10.5, E12.5, E15.5, and E18.5 and eyes from mice on postnatal day (P)0, P7, P11, P15, P30, and P60 were collected for immunohistochemical staining and in situ hybridization. One group of mice was bred strictly in the dark between E18.5 and P15, and the eyes were collected at P0, P7, P11, and P15 time points.

Results. Sp1 expression was observed in the ectoderm and lens vesicle as early as E10.5. Both Sp1 protein and mRNA were abundant in the corneal basal epithelium and keratocytes until P11. Their levels were markedly reduced at P15, right after eyelid opening, and declined further between P15 and P60. In those mice bred in the dark, Sp1 was evident in the cornea at P0. The Sp1 level gradually increased until P11 and was decreased at P15. This expression pattern was nearly identical in mice bred either in a light/dark cycle or in the dark. The Sp1 level in the central lens epithelium was much lower than that in the cornea from E15.5 to late stages.

Conclusions. The present study indicates that Sp1 expression is developmentally regulated, providing a basis for further investigations on the regulation of the *Sp1* gene during the course of corneal development and in diseases such as keratoconus. (*Invest Ophthalmol Vis Sci.* 2005;46:4092–4096) DOI:10.1167/iovs.05-0324

Sp1, one of the first eukaryotic transcription factors identified, was originally cloned as a factor that binds to the SV40 early promoter.[1,2] It is the founding member of a growing family of proteins with highly homologous zinc-finger domains close to the C terminus.[3–6] The three zinc fingers bind GC or GT boxes in the promoter or enhancer region[7] of many genes. Ubiquitously expressed,[8] this nuclear protein has been implicated in the activation or suppression of a large number of genes and is shown to be involved in cellular process such as cell cycle regulation,[9–11] chromatin remodeling,[12,13] prevention of CpG island methylation,[14,15] and apoptosis.[11,16–18] *Sp1* knockout mice further revealed that *Sp1* is essential for normal mouse embryogenesis. All $Sp1^{-/-}$ mice die at approximately day 11 of gestation.[19]

Keratoconus is a noninflammatory disease characterized by thinning, scarring, and the eventual protrusion of the central portion of the cornea.[20–23] Research in our laboratory has demonstrated that Sp1 expression is upregulated in the epithelium and stroma of the cornea,[24] but not in the conjunctiva of keratoconus patients.[25] Immunohistochemical and biochemical studies have shown that the defect of keratoconus may lie in an abnormality in degradation processes.[26,27] Degradative enzymes, including cathepsins B and G, are upregulated in keratoconus,[28–30] and protease inhibitors such as α1-proteinase inhibitor (α1-PI) are downregulated.[31,32] Our laboratory has identified multiple Sp1 binding sites in the promoter region of the human *α1-PI* gene.[33] Furthermore, overexpression of Sp1 in cultured human corneal fibroblasts and epithelial cells has been shown to repress the *α1-PI* promoter activity[33,34] but not the activity of other keratoconus-affected genes. These findings suggest that the Sp1-mediated downregulation of the *α1-PI* gene may be a key event leading to the increased degradation and pathologic condition of keratoconus corneas.

In terms of the mechanism by which *Sp1* itself is regulated, transcriptional regulation of the human *Sp1* has been shown to depend on the relative amount of Sp1, Sp3, E2F, and NF-Y in vivo.[35] Gene expression in tissues is presumably influenced by developmental and environmental factors.[36] To gain insight into the developmental regulation of Sp1, the present study was undertaken to examine the spatial and temporal expression of Sp1 in the mouse cornea throughout developing stages.

Materials and Methods

Mouse Preparation

C57BL/6 mice were raised within the Biological Resources Laboratory of the University of Illinois at Chicago (UIC) with a 14-hour light cycle and a standard chow diet. Mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocol was approved by the UIC animal care committee. For timed pregnancies, the day on which plugs were observed was considered the first day of gestation. Because diurnal variations are known to occur in the expression of transcription factors such as CLOCK and BMAL1,[37] all animals were killed between 9 and 11 AM. Embryos or eyes were collected on embryonic day (E)10.5, E12.5, E15.5, and E18.5 and on postnatal day (P)0, P7, P 11, P15, P30, and P60.

To assess the environmental effect on the Sp1 expression in the developing cornea, one group of mice was bred strictly in the dark between E18.5 and P15. Eyes were collected on P0, P7, P11, and P15 and were fixed as described below with minimal exposure to green-free light.

Immunohistochemical Analysis

Embryos or eyes were fixed at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 6 hours and were processed for paraffin

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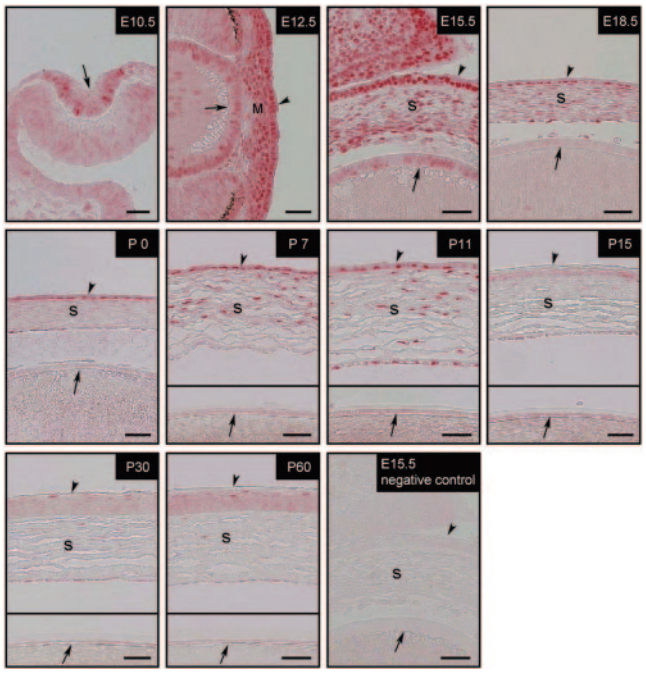

FIGURE 1. Immunostaining for Sp1 in the developing mouse cornea and lens. Sp1 staining (*pink*) was observed as early as E10.5 in the lens pit (future lens epithelium). At E12.5, Sp1 expression was abundant in the ectoderm (future corneal epithelium) and mesenchyme (future corneal stroma and endothelium). Moderate to strong nuclear staining in the basal corneal epithelium and stroma was seen between E15.5 and P11. It was dramatically reduced at P15 and then gradually declined with further maturation. A few superficial corneal epithelial cells were highly stained from P30 to P60. The expression in the lens epithelium, by comparison, was low at all stages. A serial section of the E15.5 specimen was incubated with normal rabbit IgG instead of anti-Sp1 as a negative control. *Arrow*: lens pit at E10.5 and the lens epithelium at subsequent stages; *arrowhead*: ectoderm at E12.5 and the corneal epithelium at later time points. M, mesenchyme; S, corneal stroma. Bar, 50 μm.

sections.[38] Four series of paraffin sections for each time point were prepared.

For immunohistochemistry, 5-μm–thick paraffin-embedded sections were deparaffinized, rehydrated, and incubated at room temperature with polyclonal rabbit anti-Sp1 (PEP 2) antibody (diluted 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) for 90 minutes. Biotinylated goat anti–rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody incubation at room temperature for 45 minutes. The color reaction for anti-Sp1 was carried out (Fast Red TR/Naphthol AS-MX Phosphate; Sigma, St. Louis, MO) for 5 minutes after the sections were incubated with alkaline phosphatase-conjugated Extravidin (1:50; Sigma). After mounting in aqueous mounting fluid, the staining intensity was scored by three masked observers. Minus signs indicated no staining, and plus signs indicated very faint to increasingly intense staining. For each embryonic and postnatal time point, four and three sets of paraffin sections were used to examine the levels of Sp1 in the 14-hour light cycle and in the dark, respectively. Experiments were repeated 3 times.

In Situ Hybridization

Paraffin-embedded sections were deparaffinized and rehydrated. Sections were digested at 37°C with proteinase K (10 μg/μL; Promega) for 12 minutes and postfixed at 4°C with 1% paraformaldehyde in diethyl pyrocarbonate (DEPC)–treated water for 5 minutes. Slides were hybridized at 42°C overnight with digoxigenin-labeled Sp1 cDNA probe (1.5 μg/mL) prepared using a labeling kit (PCR DIG; Roche, Indianapolis, IN). These probes were synthesized from the total RNA isolated from C57BL/6J mouse eyes using target-specific primers (Sp1, 5′-GGCAATAATGGGGGTAGCGG-3′ and 5′-CAAGCTGGCAGAACTGATGGC-3′). Tissues were further incubated with alkaline phosphatase-conjugated anti-digoxigenin (Roche), and the color was developed by reaction with NBT/BCIP (Roche). The sections were mounted, and the staining was scored as described. Two sections for each embryonic and postnatal time point were included, and experiments were repeated 3 times.

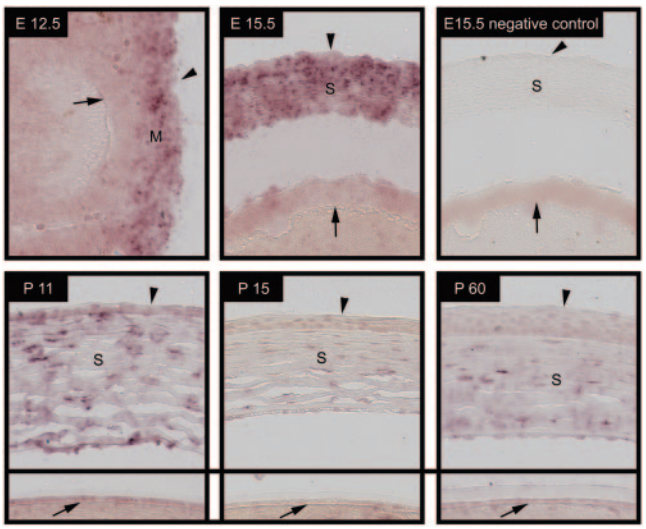

FIGURE 2. In situ hybridization for Sp1 in the developing mouse cornea and lens. *Purplish blue* hybridization products for Sp1 transcript were detected in the ectoderm and mesenchyme at E12.5. The transcript was also found in the corneal epithelium and stroma from E15.5 until P11. It was then reduced at P15 in the basal corneal epithelial cells and keratocytes and remained weak thereafter until P60. In the lens epithelium, the Sp1 transcript was scanty between E15.5 and P60. A serial section of the E15.5 specimen was incubated with reagent mixture without the cDNA probe as a negative control. *Arrow*: lens epithelium; *arrowhead*: ectoderm at E12.5 and corneal epithelium at later time points. M, mesenchyme; S, corneal stroma.

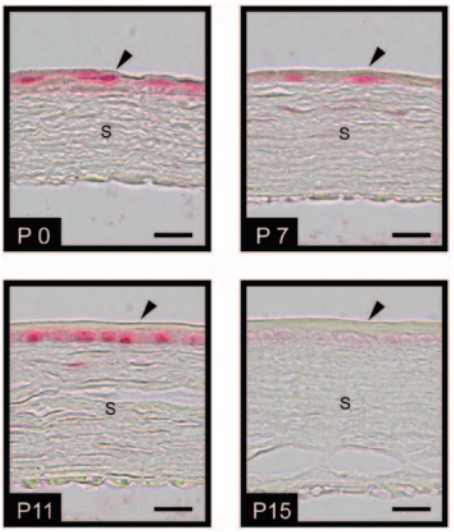

FIGURE 3. Immunostaining for Sp1 in mice bred in the dark. In these mice, Sp1 protein expression was evident in basal corneal epithelial cells and keratocytes at P0, P7, and P11. It was found much reduced at P15. This pattern of Sp1 expression was nearly identical with that observed in the mice bred in the 14-hour light cycle shown in Figure 1. *Arrowhead*: corneal epithelium. S, corneal stroma. Bar, 50 μm.

TABLE 1. Immunostaining and In Situ Hybridization Patterns of Sp1

	E 10.5	E 12.5	E 15.5	E 18.5	P 0	P 7	P 11	P 15	P 30	P 60
Immunostaining										
Corneal epithelium	N/A	+++	+++	+++	+++	+++	+++	+	±	±
Corneal stroma	N/A	+++	+++	++	++	+++	+++	+	+	+
Corneal endothelium	N/A	N/A	N/A	N/A	±	±	+~++	±	±	±
Lens epithelium	++	++	+	±	−~±	−~±	−~±	−~±	−~±	−~±
In situ hybridization										
Corneal epithelium	N/A	++	+++	++	++	++	++	+	±	±
Corneal stroma	N/A	++	+++	++	++	++	++	+	+	+
Corneal endothelium	N/A	N/A	N/A	N/A	−	±	+~++	±	±	±
Lens epithelium	+	+	±	−~±	−~±	−~±	+	−~±	−~±	−~±

Staining intensity for Sp1 protein or transcript was graded under a light microscope by three masked observers. The range of scores is presented. Minus signs indicate no staining, plus/minus signs indicate very faint staining, and "+" to "+++" indicates increasingly intense staining.

RESULTS

By immunohistochemical staining, the presence of Sp1 was evident in the lens pit (future lens epithelium) as early as E10.5 (Fig. 1, E10.5). At E12.5 (Fig. 1, E12.5), Sp1 expression in the nuclei was abundant in the ectoderm (future corneal epithelium) and mesenchyme (future corneal stroma and endothelium). The Sp1 level at E15.5 and E18.5 (Fig. 1, E15.5 and E18.5) remained relatively high in the corneal epithelium and stroma, and the robust expression in the cornea continued until P11 (Fig. 1, P11). Notably, the expression of Sp1 in basal corneal epithelial cells and keratocytes was much reduced at P15 (Fig. 1, P15), right after eyelid opening, which occurred at P13 or P14. The Sp1 level in both types of cells further declined between P15 and P60, whereas a few superficial corneal epithelial cells were found moderately to strongly positive for anti-Sp1 from P30 to P60 (Fig. 1, P30 and P60). In the corneal endothelium, the Sp1 staining pattern was different from that of corneal epithelial cells and keratocytes. The protein was observed at P0, and the level remained low until P7. It was increased to a moderate level at P11 but was reduced again at P15 and remained low afterward (Fig. 1, P0-P60).

Staining of Sp1 in the central lens epithelium was much lower than that in the cornea. The highest level in the lens epithelium was noted at E12.5. After E15.5, Sp1 levels continued to diminish until nearly absent at postnatal stages (Fig. 1, E10.5-P60).

In situ hybridization experiments detected positive hybridization products for Sp1 transcript in the lens pit at E10.5 (data not shown) and in the ectoderm and mesenchyme at E12.5 (Fig. 2, E12.5). The Sp1 transcript was also found in the corneal epithelium and stroma at E15.5 (Fig. 2, E15.5). The high level of Sp1 mRNA in these corneal layers continued until P11 (Fig. 2, P11). It was then noticeably reduced at P15 in the basal corneal epithelial cells and keratocytes and remained low until P60 (Fig. 2, P15-P60). In the endothelium, Sp1 mRNA was observed at P7, and the strongest staining was seen at P11. It was diminished to a low level at P60. In the lens epithelium, the transcript of Sp1 was barely detectable throughout the stages between E15.5 and P60 (Fig. 2, E15.5-P60).

Staining intensity for Sp1 in the basal corneal epithelium, stroma, and endothelium and the lens epithelium at each developmental stage point was scored. The range of scores is summarized in Table 1. Both immunostaining and in situ hybridization showed a declining Sp1 expression pattern.

In mice bred in the dark, Sp1 immunostaining was evident in basal corneal epithelial cells and keratocytes at P0 (Fig. 3, P0). The moderate to strong expression continued until P11 (Fig. 3, P7 and P11). It was, however, found much reduced at P15 (Fig. 3, P15). This pattern of Sp1 expression was nearly identical with that observed in the mice bred in a 14-hour light cycle, shown in Figure 1.

DISCUSSION

The present study demonstrates that the expression of transcription factor Sp1 in the mouse cornea is developmentally regulated. Immunohistochemical studies indicated that the Sp1 protein in the cornea, especially in corneal basal epithelial cells and keratocytes, was abundant until P11. It was dramatically decreased at P15 and remained low in the adult mouse cornea except in a few superficial epithelial cells. By contrast, the Sp1 level in the central lens epithelium was very weak from E18.5 to all the late stages. Sp1 might not have as important a role in the late lens differentiation as other transcription factors, such as Krüppel-like factor 6.[39] It is of note nonetheless that the Sp1 expression pattern in the lens and the corneal epithelium is different, even though both are derived from the ectoderm between E10.5 and E12.5. These observations are consistent with results from previous developmental[8] and *Sp1* null mouse[19] studies that the expression of Sp1 differs in various cell types during development and that, aside from its general role in the transcription of housekeeping genes, Sp1 has an important regulatory function in development. Also in agreement with previous findings,[8] our data show that Sp1 is downregulated in fully differentiated cells, such as corneal epithelial cells and keratocytes, of adult mouse and in the nondividing and essentially quiescent central epithelial cells[40] of the lens.

Sp1 in the corneal basal epithelium and keratocytes exhibits a dramatic decrease from P11 to P15 that seems to coincide with eyelid opening. This result suggests that developmental programming or environmental changes caused by eyelid opening, including altered light and increased oxidative stress,[36,41] may influence Sp1 expression. To evaluate the effect of light on Sp1 expression, one group of mice was bred in the dark. In these mice, the Sp1 level in the cornea at P15 was still much lower than that at P11. Therefore, we concluded that the altered light might not have had much influence on Sp1 expression during corneal development. Further investigation into the impact of oxidative stress and other factors on the Sp1 expression is merited.

The developmental pattern of Sp1 mRNA transcript in the mouse cornea, as revealed by in situ hybridization, paralleled that of the protein expression of Sp1. Sp1 protein has been shown to be related to a large extent to the mRNA level,[8] but there are also other control mechanisms, such as proteasome-dependent degradation.[42,43] Our data suggest that Sp1 protein in the mouse cornea is mostly regulated at the transcriptional level.

In the adult mouse cornea (Fig. 1, P30 and P60), strong Sp1 expression was observed in a few superficial epithelial cells. Epithelial renewal is a constant feature of the corneal epithelium in homeostasis.[44] Precisely how cells are lost from the corneal epithelium has not been fully clarified, though Ren and Wilson[45] suggest that apoptosis[46] is involved. Fas ligand (FasL)

is a key physiologic inducer of apoptosis.[47] It was reported that FasL mRNA is expressed in primary cultures of human corneal fibroblasts, epithelial cells, and endothelial cells.[48] FasL protein was detected in the epithelial and endothelial cells in fresh-frozen human corneas,[48] and it was also noted in keratocytes under diseased conditions such as in Fuchs' corneal dystrophy[49] and keratoconus (Nakamura H, et al., unpublished observation, 2003). Recently, evidence has been provided that Sp1 upregulates apoptosis through the promoter region of FasL and caspase 3 in differentiated cells including Sertoli, Jurkat, T, smooth muscle, and PC12 cells.[11,16–18,50] We thus speculate that Sp1 expression may be related to the apoptotic process in the superficial corneal epithelial cells.

For more quantitative data, we attempted Western blot and relative quantitative RT-PCR analyses at least three times using the developing corneal samples (data not shown). However, the results did not correlate well with those of the immunohistochemical analysis and in situ hybridization. Samples used for quantitative analyses were from full-thickness corneas because it was difficult to dissect each corneal layer separately. It is thus reasoned that the inclusion of corneal endothelial cells and superficial epithelial cells that showed intense Sp1 expression might have presented a complicated scenario. Isolation of single cells from different layers of corneal tissues seems to be necessary, and recently developed techniques such as laser capture microdissection may aid in this effort.

Although the mechanism is unclear, Sp1 has been shown to be upregulated in keratoconus. Results of the present study imply that perhaps in keratoconus, the postdevelopmental program engineered to silence Sp1 is aberrant such that the Sp1 level in the mature cornea remains abnormally high or unsuppressed. The human Sp1 promoter has been cloned and characterized,[51] and the 1.6-kb promoter is GC rich. The presence of CpG islands suggests the possibility that epigenetic mechanisms such as DNA methylation[52–54] and histone acetylation[53,55] may be involved in the transcriptional regulation of Sp1.

In summary, we presented herein a dynamic pattern of developmental expression of transcription factor Sp1 in the mouse cornea. The results provide a basis for further investigation into the regulation of the *Sp1* gene during corneal development and in diseases such as keratoconus.

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