



Epithelial SOX11 regulates eyelid closure during embryonic eye development

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ABSTRACT

Fibroblast growth factor (FGF10)-mediated signals are essential for embryonic eyelid closure in mammals. Systemic SOX11-deficient mice are born with unclosed eyelids, suggesting a possible role of SOX11 in eyelid closure. However, the underlying mechanisms of this process remain unclear. In this study, we show that epithelial deficiency of SOX11 causes a defect in the extension of the leading edge of the eyelid, leading to failure of embryonic eyelid closure. c-Jun in the eyelid is a transcription factor downstream of FGF10 required for the extension of the leading edge of the eyelid, and c-Jun level was decreased in epithelial SOX11-deficient embryos. These results suggest that epithelial SOX11 plays an important role in embryonic eyelid closure.

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1. Introduction

Embryonic eyelid closure is common to all mammals and regulated by various biological processes [1]. The ectoderm layer around the mesoderm forms a protuberant of epithelium, referred to as the eyelid root [2], which subsequently extends to fuse with the eyelid root of the opposing eyelids. Impaired closure of the embryonic eyelid causes the eye open at birth (EOB) phenotype in mice. Previous studies suggested that fibroblast growth factor (FGF) 10 [3] and its receptor, FGFR2B, play an important role in embryonic eyelid closure [4,5]. More than one hundred EOB mouse strains have been reported (publicly annotated by Mouse Genome Informatics: <http://www.informatics.jax.org>). In the public database, mice deficient in bone morphogenetic protein (BMP) 4, activin β B, heparin-binding (HB)-epidermal growth factor (EGF), transforming growth factor (TGF) α and c-Jun are annotated as EOB mouse strains; all these factors are the components of the signals downstream of FGF10 and FGFR2B [6,7]. These findings collectively suggest that many EOB mouse strains have defects in the signaling

pathways related to FGF10 and FGFR2B.

FGF10 is produced by the developing eyelid mesenchyme [3] and stimulates the downstream signals via FGFR2B in eyelid epithelial cells [1]. Stimulation of FGFR2B by FGF10 activates various signaling pathways in the eyelid epithelium: (i) BMP4-BMPR1/2-SMAD4-mediated translocation of phosphorylated c-Jun from the cytosol to the nucleus [6,8]; (ii) activin β B-ActivinR1/2-JNK-mediated c-Jun phosphorylation [7,9,10]; and (iii) ligands of EGF receptor such as HB-EGF and TGF α -EGFR-mediated ERK phosphorylation [2,11]. Activated ERK and c-Jun coordinately promote actin fiber polymerization in epithelial cells at the leading edge of the eyelid root [2]. Various EOB mouse strains have been previously identified; however, it remains unknown whether the deletion of these previously reported signaling molecules causes EOB phenotypes via interference with the FGF10-FGFR2B signaling pathways that regulate epithelial cell migration and eyelid closure.

SRY-related HMG box (SOX) 11 is a transcription factor that belongs to the SOXC family together with SOX4 and SOX12. During embryonic eyelid development, SOX11 is expressed in the mesenchyme and epithelium of the eyelid roots in E14 embryos [12,13]. An earlier study revealed that systemic *Sox11*-deficient mice are born with EOB phenotypes [14]. These findings suggest that SOX11 is a transcription factor that plays an important role in closure of the embryonic eyelid in mice. However, these mice immediately die after birth, probably due to heart defects [14]. Thus, the mechanisms of regulation of eyelid closure by SOX11 are poorly understood. It is not known whether SOX11 is involved in the FGF10-

Abbreviations: BMP, bone morphogenetic protein; EOB, eye open at birth; EGF, epidermal growth factor; FGF, fibroblast growth factor; HB, heparin-binding; Krt, keratin; PCNA, proliferating cell nuclear antigen; SOX, SRY-related HMG box; TGF, transforming growth factor.

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FGFR2B signaling pathways and whether SOX11 in either mesenchyme or epithelium contributes to embryonic eyelid closure. Moreover, it remains unclear how the EOB phenotypes induced by SOX11 deficiency affect ocular surface state in postnatal mice. A previous study reported that SOX11 regulates the expression of FGFR2 in the epithelium of the developing palatal shelf [15], whereas no report has shown the involvement of SOX11 in the mesenchymal expression of FGF10. Moreover, SOX11 is involved in the activation of BMP2/4-SMAD4 signaling during bone development [16,17]. These findings suggest that SOX11 may be involved in the FGFR2B-mediated signal cascades, specifically including the BMP4-BMPRI1/2-SMAD4-c-Jun pathways in developing eyelid epithelium, rather than in the FGF10 production by the mesenchyme.

To address this hypothesis, we determined whether deletion of SOX11 in the eyelid epithelium causes impaired eyelid closure by interfering with the activation of FGFR2B-mediated signaling pathways in the eyelid epithelium. In the present study, we generated epithelial *Sox11*-deficient mice by crossing *Sox11^{fl/fl}* mice with *keratin (Krt) 5-cre* transgenic mice to determine the role of SOX11 in the epithelium in eyelid closure. Here, we show that ablation of epithelial SOX11 caused defects in the embryonic eyelid closure process and corneal opacity in postnatal mice; additionally, we investigated the contribution of SOX11 to embryonic eyelid closure regulated by the signaling molecules related to FGF10 and FGFR2B.

2. Materials and Methods

2.1. Study approval

All procedures for the animal experiments were approved by the Saga University Animal Care and Use Committee.

2.2. Generation and care of mice

Sox11^{fl/fl} mice [18] were generated by Dr. Véronique Lefebvre at the Cleveland Clinic Foundation. *Krt5-cre* mice [19] were provided by the Center for Animal Resources and Development (Kumamoto University). *R26^{tdRFP}* mice [20,21] were prepared by Dr. Hans Joerg Fehling. All mice were used on the C57BL/6 background. These mice were bred in the animal facility of the Saga Medical School under specific pathogen-free conditions.

2.3. Mouse genotyping

The PCR conditions for *Sox11^{fl/fl}* included the initial denaturation step at 95 °C for 10 min, followed by 30 cycles at 94 °C for 30 s, 65 °C for 40 s, and 72 °C for 60 s with the final extension step at 72 °C for 5 min. For *Krt5-cre* amplification, the conditions included denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 60 s, 58 °C for 120 s, and 72 °C for 120 s with the final extension step at 72 °C for 10 min. The primers were as follows: *Sox11^{fl/fl}* Fwd (5'-TTCGTGATTGCAACAAAGGCGGAG-3') and *Sox11^{fl/fl}* Rev (5'-GCTCCCTGCAGTTTAAGAAATCGG-3') and *Krt5-cre* Fwd (5'-AGGTTCTGTTCACTCATGGA-3') and *Krt5-cre* Rev (5'-TCGACCAGTT-TAGTTACCC-3').

2.4. Immunohistochemistry

Immunostaining with SOX11 antibodies (1:200; Atlas Antibodies, Bromma, Sweden or 1:200; Merck Millipore, Burlington, MA, USA) was performed using 10% formalin- or 4% paraformaldehyde-fixed tissues. The fixed tissues samples were incubated with primary antibodies at the indicated dilution. An

EnVision system (Agilent Technologies, Santa Clara, CA, USA) was used to detect the colorimetric signals, and the samples were then counterstained with hematoxylin. The color images were deconvoluted using Image J software. For morphological studies, the sections were stained with hematoxylin and eosin (H&E).

2.5. Immunofluorescence detection by confocal microscopy

Tissue specimens were stained with anti-PCNA (1:200; Dako/Agilent Technologies, Santa Clara, CA, USA), anti-phospho-c-Jun (1:200; Abcam, Cambridge, UK), anti-c-Jun (1:100; Santa Cruz Biotechnology, Dallas, TX, USA), or anti-phospho EGFR (1:200; Abcam). Goat anti-mouse IgG conjugated with Alexa 488 or goat anti-rabbit IgG conjugated with Alexa 546 were used to visualize the signals. Nuclei were stained with NucBlue (Thermo Fisher Scientific, Waltham, MA, USA). The sections were mounted using a fluorescent mounting medium (Agilent Technologies). F-actin was detected by phalloidin conjugated to TRITC (Vector Laboratories, Burlingame, CA, USA). The sections were analyzed using an LSM5 PASCAL G/B microscope (Carl Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

The data shown are the mean \pm standard deviation (SD) or standard error (SE). Statistical analyses were performed with Prism 5.0 software (GraphPad Software, La Jolla, CA, USA) using two-sided Mann-Whitney t-tests. *p* values less than 0.05 were considered significant.

3. Results

3.1. Defect of eye closure in *Sox11^{Δk5}* mice

We crossed *Krt5-cre* transgenic mice with *Sox11^{fl/fl}* mice to delete epithelial SOX11. *Krt5-cre⁺; Sox11^{fl/fl} (Sox11^{Δk5})* mice developed the EOB phenotype (Fig. 1A). The opened eyelids of *Sox11^{Δk5}* mice were unclosed during P0–P14. Moreover, eye opacity was observed in the *Sox11^{Δk5}* mice (P12), similar to that in other EOB mice (Fig. 1A) [7,11,22–24]. Then, we examined whether the *Krt5-cre* transgene results in the EOB phenotype and eye opacity in *Sox11^{fl/fl}* mice. The *Krt5-cre⁻; Sox11^{fl/fl}* (0%; 0 out of 30), *Krt5-cre⁻; Sox11^{fl/wt}* (0%; 0 out of 9), and *Krt5-cre⁺; Sox11^{fl/wt}* (0%; 0 out of 12) mice did not manifest the EOB phenotype and eye opacity; however, the *Krt5-cre⁺; Sox11^{fl/fl}* mice (100%; 33 out of 33) manifested both phenotypes (Supplemental Table 1). These results indicated that the incidence of EOB and eye opacity are due to the homozygous deletion of SOX11 in the epithelium by the *Krt5-cre* transgene. *Sox11^{Δk5}* mouse pups grew normally (Fig. 1B). There were no significant differences in the body weight between 8-week-old *Sox11^{Δk5}* and *Sox11^{fl/fl}* mice (Fig. 1C). However, *Sox11^{Δk5}* adult mice had small eyeballs and exhibited microphthalmia of variable degree (Supplemental Fig. 1). For example, 66.67% (22 out of 33) of *Sox11^{Δk5}* adult mice had mild microphthalmia in both eyes. *Sox11^{Δk5}* adult mice manifested severe microphthalmia in both eyes (6.06%, 2 out of 33) or in one eye (left eye: 18.18%, 6 out of 33; right eye: 9.09%, 3 out of 33). These data suggest that epithelial deletion of SOX11 by *Krt5-cre* had no effect on whole body growth but interfered with the processes related to eye formation.

3.2. Corneal inflammation in the eyes of adult *Sox11^{Δk5}* mice

Some adult EOB mice were reported to develop corneal inflammation characterized by keratitis with corneal opacity [7,11,22–24]. Thus, we examined whether the clouded eyes of adult *Sox11^{Δk5}* mice are due to corneal inflammation. The adult *Sox11^{Δk5}*

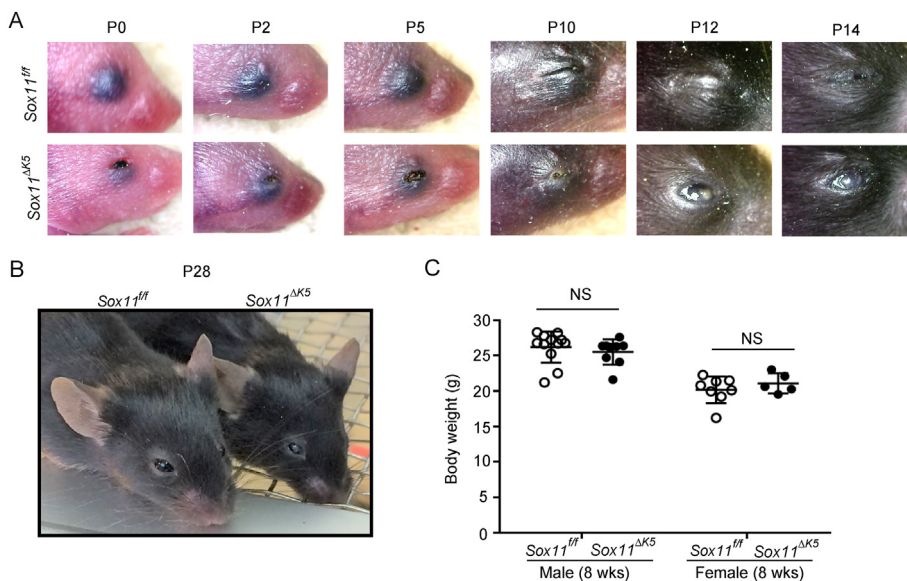


Fig. 1. *Sox11*^{ΔK5} mice show open eyelids at birth and eye opacity (A and B) The appearances of *Sox11*^{ΔK5} and *Sox11*^{fl/fl} mice. Postnatal days 0, 2, 5, 10, 12, 14 (A), and 28 (B). (C) Normal growth of the whole body. Body weight of adult *Sox11*^{ΔK5} and *Sox11*^{fl/fl} mice was measured at 8 weeks (male *Sox11*^{ΔK5}, n = 9; male *Sox11*^{fl/fl}, n = 12; female *Sox11*^{ΔK5}, n = 5; female *Sox11*^{fl/fl}, n = 8). The data shown are the mean ± SE. Statistical analysis was performed using a two-sided Mann-Whitney *t*-test.

mice showed corneal opacity with neovessels (Fig. 2A). H&E staining indicated the formation of stromal neovessels and epithelial hyperplasia in the corneas of *Sox11*^{ΔK5} mice (Fig. 2B).

These data show that adult *Sox11*^{ΔK5} mice develop keratitis with corneal opacity.

3.3. Impaired elongation of the eyelid roots in *Sox11*^{ΔK5} embryos

During eyelid closure in mouse embryos, the epithelium at the leading edge of the eyelid elongates and migrates to fuse the opposing eyelids from E15 to E16.5 [2]. Therefore, we examined whether embryonic eyelid closure is impaired in *Sox11*^{ΔK5} embryos. As expected, eyelid closure was detected in *Sox11*^{fl/fl} E15.5 embryos but not in *Sox11*^{ΔK5} E15.5 embryos (Fig. 3A). The length of the initially formed eyelid roots was similar in *Sox11*^{fl/fl} and *Sox11*^{ΔK5} E14 embryos; however, the root length of *Sox11*^{ΔK5} embryos was shorter than that of *Sox11*^{fl/fl} embryos at E15.5 (Fig. 3B). These data suggest that SOX11 plays an essential role in the extension of the eyelid roots. SOX11 immunostaining indicated that the eyelid root mesenchyme regions of the *Sox11*^{ΔK5} and *Sox11*^{fl/fl} E15.5 embryos expressed similar levels of SOX11 (Fig. 3C). In contrast, there was an apparent difference in the eyelid epithelium and palpebral conjunctiva. The epithelium at the leading edge and palpebral conjunctiva of the eyelid of the *Sox11*^{fl/fl} embryos expressed SOX11 in the nuclei. Epithelial expression of SOX11 was barely detectable in the *Sox11*^{ΔK5} embryos. These results suggest that SOX11 expression in the leading edge and palpebral conjunctiva of the eyelid is essential for the initiation of embryonic eyelid closure.

3.4. Filamentous (F)-actin accumulation in the leading edge of the eyelid is impaired in the *Sox11*^{ΔK5} embryos

Previous studies have shown that normal eyelid closure requires keratinocyte proliferation in the basal layer of the eyelid root [3,25]. Thus, we examined whether SOX11 ablation affects epithelial cell proliferation in the eyelids. To address this issue, we performed proliferating cell nuclear antigen (PCNA) staining. The numbers of PCNA⁺ basal cells in the epithelium of the eyelid roots were comparable in the *Sox11*^{ΔK5} and *Sox11*^{fl/fl} E15.5 embryos (Fig. 4A). This result suggests that SOX11 depletion did not influence cell proliferation in the eyelid roots. During eyelid closure, filamentous (F)-

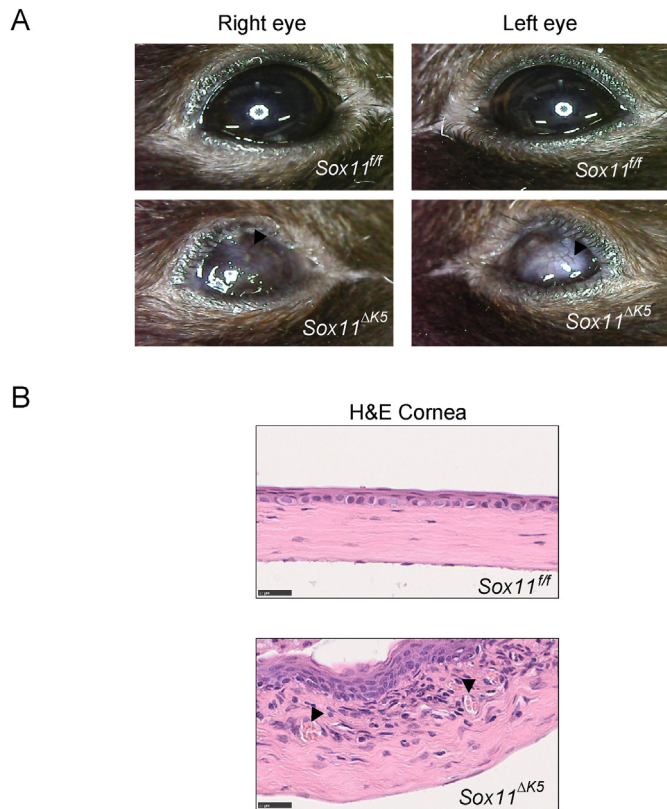


Fig. 2. Corneal opacity and neovessel formation in the eyes of adult *Sox11*^{ΔK5} mice (A) Corneal opacity and neovessel formation in adult *Sox11*^{ΔK5} mice. Black arrowheads indicate neovessels on the cornea. (B) Histological analysis of the eyes. H&E staining. Black arrowheads indicate neovessels in the corneal stroma. Scale bars: 25 μm.

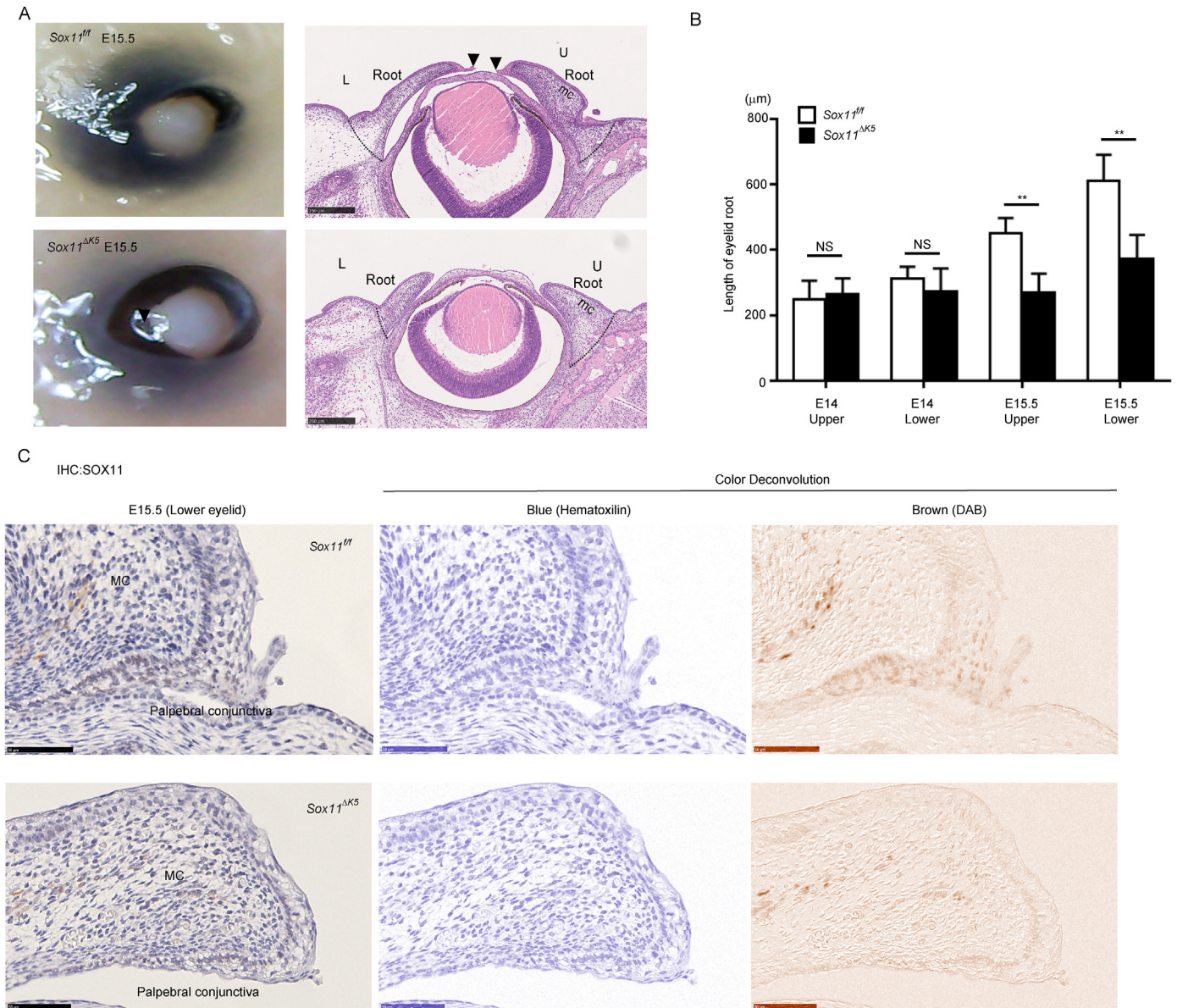


Fig. 3. SOX11 is required for eyelid closure during embryonic eye development

(A) Impaired eyelid closure in the *Sox11^{ΔK5}* E15.5 embryos. H&E staining of the *Sox11^{ΔK5}* and *Sox11^{fl/fl}* E15.5 embryos (right panels). Eyelid root boundary was defined by the line between the base of the eyelid and conjunctiva fornix. Black arrowheads and dashed lines indicate the leading edge of the eyelid and root boundary, respectively. L, lower eyelid. U, upper eyelid. MC, mesenchyme. (B) Length of the eyelid roots in the E14 and E15.5 embryos. The data shown are the mean \pm SD (E14 *Sox11^{fl/fl}*, n = 4; E14 *Sox11^{ΔK5}*, n = 2; E15.5 *Sox11^{fl/fl}*, n = 6; E15.5 *Sox11^{ΔK5}*, n = 11). Statistical analysis was performed using a two-sided Mann-Whitney *t*-test; ***p* < 0.01 versus *Sox11^{fl/fl}*. NS, not significant. (C) Lack of epithelial SOX11 in the eyelids of *Sox11^{ΔK5}* E15.5 embryos. SOX11 immunostaining of the *Sox11^{ΔK5}* and *Sox11^{fl/fl}* E15.5 embryos. Images of color deconvolution are depicted (center and right panels). Scale bars: 250 μ m (A) and 50 μ m (C). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

actin accumulation in the eyelid leading edge is essential for the migration of eyelid epithelial cells [7,10]. F-actin accumulation in the eyelid leading edge was detected in the *Sox11^{fl/fl}*, but not *Sox11^{ΔK5}*, E15.5 embryos (Fig. 4B), indicating that SOX11 might be involved in the regulation of F-actin formation.

3.5. SOX11 regulates the expression of the c-Jun protein in the eyelid

BMP4-SMAD4-mediated translocation of phosphorylated c-Jun from the cytosol to the nucleus, activin β B-JNK-mediated c-Jun phosphorylation and TGF α /HB-EGF-ERK activation are essential for the migration of the leading edge of the eyelid [1,2,6,7,10,11].

Therefore, we examined whether SOX11 deficiency reduces the number of the eyelid cells containing phosphorylated c-Jun in the nuclei. The number of positive cells of phosphorylated c-Jun were significantly decreased in the nuclei of the leading edge and palpebral conjunctiva of the eyelid of the *Sox11^{ΔK5}* embryos (Fig. 4C). Similarly, c-Jun expression was locally reduced in the leading edge and palpebral conjunctiva of the eyelid of the *Sox11^{ΔK5}* embryos. In contrast, SOX11 deficiency did not reduce EGFR phosphorylation, implying that the TGF α /HB-EGF-ERK signals via EGFR are not impaired in the *Sox11^{ΔK5}* E15.5 embryos (Fig. 4D). These data suggest that epithelial SOX11 may play important roles in the expression of the c-Jun protein during eyelid closure.

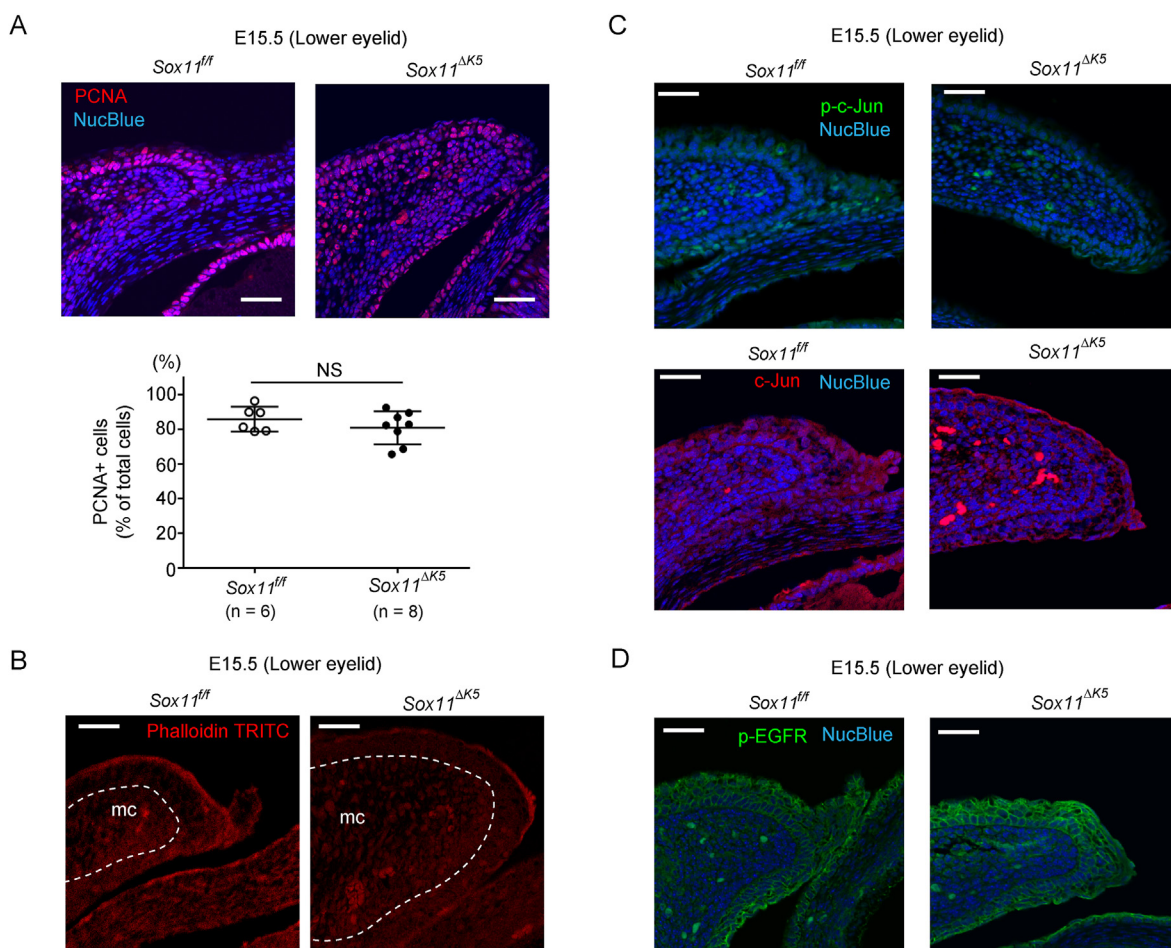


Fig. 4. SOX11 is involved in F-actin accumulation and c-Jun expression during eyelid closure

(A) Comparable epithelial cell proliferation of the eyelid roots of the *Sox11*^{ΔK5} and *Sox11*^{fl/fl} embryos. PCNA immunofluorescence staining of the *Sox11*^{ΔK5} and *Sox11*^{fl/fl} E15.5 embryos is depicted (upper panels). Quantitative analysis of PCNA staining (bottom panel) (B) Decreased F-actin accumulation in the leading edge of the eyelid of the *Sox11*^{ΔK5} E15.5 embryos. The *Sox11*^{ΔK5} and *Sox11*^{fl/fl} E15.5 embryos were stained with phalloidin-TRITC. mc, mesenchyme. (C and D) Immunofluorescence staining for phosphorylated c-Jun (Ser73) (Green, C upper panels), c-Jun (Red, C lower panels), and phospho EGFR (Tyr1068) (Green) in the *Sox11*^{ΔK5} and *Sox11*^{fl/fl} E15.5 embryos. Scale bars: 100 μm (A) and 50 μm (B–D). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

EOB phenotypes have been reported to be associated with numerous genotypes; currently, the Mouse Genome Informatics database lists 164 mouse strains that display EOB phenotypes. Systemic *Sox11*-deficient mice are included in the list. A previous study reported the EOB phenotypes of *Sox11*-deficient mice [18]; however, the mechanism of induction of EOB in *Sox11*-deficient mice is unclear. In the present study, we show that epithelial deletion of the *Sox11* gene by *Krt5-cre* causes EOB phenotypes by impairing eyelid closure. SOX11 was expressed at a high level in the leading edge of the developing eyelid of E15.5 embryos. Consistent with the expression pattern of this gene, the formation of the leading edge of the eyelid was severely impaired in the *Sox11*^{ΔK5} E15.5 embryos. We then demonstrate that F-actin accumulation, which is required for epithelial cell migration, was decreased in the eyelid tip of the *Sox11*^{ΔK5} E15.5 embryos. c-Jun plays an essential role in polymerization of actin fiber [6,7,10,11], and the expression levels was significantly decreased in the eyelids of the *Sox11*^{ΔK5} E15.5 embryos. Our findings suggest that SOX11 may act upstream of the c-Jun-dependent cellular events in eyelid closure.

EOB phenotypes and postnatal corneal opacity are two main features associated with epithelial SOX11 deficiency that are

characterised by complete penetrance (Supplemental Table 1). Histological analysis of the clouded eyes of adult *Sox11*^{ΔK5} mice detected several abnormalities, such as stromal neovascularization and thickening of the corneal epithelium and stroma (Fig. 2B) similar to the characteristics of other EOB phenotypes [11,23,26]. Anti-vascular endothelial growth factor therapy reduces neovessel formation in the cornea and improves corneal opacity [27]; hence, we believe that corneal opacity in *Sox11*^{ΔK5} mice is also due to corneal neovascularization. The mechanism of the obligatory development of corneal neovascularization in EOB mouse strains, including *Sox11*^{ΔK5}, has not been elucidated; however, ocular surface hypoxia might promote angiogenesis. Previous reports showed that dry eye- or contact lens wear-related ocular surface hypoxia promotes neovascularization [28] and that EOB mice have defects in the development of the Meibomian gland, which provides tears containing oxygen [22]. Additional analysis of the Meibomian gland formation and ocular surface hypoxia is required to determine the mechanisms of the development of corneal neovascularization and opacity in *Sox11*^{ΔK5} mice.

Two distinct cellular processes, including the proliferation of epithelial cells of the eyelid root and migration of epithelial cells of the leading edge, are essential for eyelid closure [3,25]. The results of the present study indicate that epithelial SOX11 is required for

the migration of epithelial cells, but not for their proliferation, during embryonic eyelid closure (Fig. 4A). Our data show actin fiber polymerization in the migrating eyelid epithelium in the *Sox11^{fl/fl}* E15.5 embryos (Fig. 4B). Since non-migrating epithelial cells in the embryonic eyelid of EOB mouse strains fail to reorganize actin fiber [1,10], we believe that SOX11 might play a pivotal role in the epithelial cell migration at the leading edge of the eyelid by promoting F-actin accumulation. Previous studies have shown that c-Jun-deficient epithelial cells lack the ability to accumulate F-actin [7,9]. Notably, the expression and phosphorylation of c-Jun were apparently decreased in the eyelid tips and palpebral conjunctiva of the *Sox11^{Δk5}* E15.5 embryos (Fig. 4C). These data suggest that downregulation of c-Jun expression induced by epithelial deletion of SOX11 may be responsible for a reduction in the number of c-Jun-positive nuclei and intensity of phosphorylated c-Jun staining in the nuclei of the eyelid cells and contributes to a defect of actin fiber polymerization. However, it is unclear whether SOX11 deficiency interferes with c-Jun phosphorylation via activin β B-JNK signaling. Future studies will be performed to investigate these phenomena. Although the precise mechanism remains unclear, we currently believe that the nuclear translocation process of phosphorylated c-Jun via BMP4-Smad4 signals is intact in *Sox11^{Δk5}* embryos because cytosolic localization of phosphorylated c-Jun was detected in *Sox11^{fl/fl}* and *Sox11^{Δk5}* embryos.

In conclusion, in this study, we demonstrate that epithelial SOX11 contributes to embryonic eyelid closure in mice. Human eyelids also fuse during fetal development, and the morphogenetic process of eyelid closure is thought to resemble that of mouse eyelids. However, human eyelid closure and reopening are completed in utero. Therefore, it is difficult to detect congenital failure of eyelid closure caused by the loss or mutation of a single gene in humans. It is unclear whether SOX11 deficiency or malfunction causes the failure of eyelid closure; however, mouse embryos require epithelial SOX11 expression to fuse the eyelids. Although further investigation will be necessary to address these issues, our findings will help elucidate the regulatory mechanisms of eyelid closure and will provide useful information on developmental defects of the eyelid closure in humans.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2021.02.075>.

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