MEK kinase 1 regulates c-Jun phosphorylation in the control of corneal morphogenesis

Lin Zhang,1,2 Maoxian Deng,1,2 Candace W.-C. Kao,3 Winston W.-Y. Kao,3 Ying Xia1,2

1Center for Environmental Genetics, 2Department of Environmental Health, and 3Department of Ophthalmology, University of Cincinnati Medical Center, Cincinnati, OH

Purpose: To study the in vivo role of MEK kinase 1 (MEKK1) in corneal development.

Methods: Wild type and Mekk1∆KD mice eye tissues were examined by staining with hematoxylin and eosin for morphogenesis and Masson’s trichrome for extracellular matrix (ECM) deposition. The cells expressing ECM gene transcripts of Collagen I, Keratan, and Lumican in corneal stroma were identified by in situ hybridization and the level of Collagen I mRNA in the developing cornea was quantified by real-time RT-PCR. Immunohistochemistry staining was employed to study the expression and N-terminal phosphorylation of c-Jun and the expression of epithelium differentiation markers and intercellular structural proteins of the corneal epithelium.

Results: Mekk1∆KD mice exhibited the “eye open at birth” phenotype (EOB), and developed eye defects and severe pathology secondary to impaired eyelid formation. The corneal stroma of Mekk1∆KD fetuses, although exhibiting normal morphology, thickness, and keratocyte proliferation, showed reduced extracellular matrix (ECM) accumulation, corresponding to a decrease in transcription of Lumican, Keratan, and Collagen I. Immunohistochemistry studies demonstrated that MEKK1 ablation caused a remarkable reduction in the expression of occludin and zonula occluden protein-1 (ZO-1), components of tight junction, but had no effect on the expression of E-cadherin and β-catenin for adherens junctions, desmoplakin and desmoglein for desmosomes and cytokeratins 12 and 14 for cornea-type epithelial differentiation in the developing cornea. c-Jun was abundantly expressed in the developing corneal epithelium and its phosphorylation was considerably reduced in Mekk1∆KD fetuses.

Conclusions: In addition to its role in eyelid morphogenesis, MEKK1 is crucial for corneal development such that its ablation caused a reduction of ECM deposition in corneal stroma and disturbance of tight junctions in corneal epithelium. c-Jun phosphorylation in corneal epithelium is a downstream event of the MEKK1 pathway, likely contributing to corneal development and function. Altogether, MEKK1 plays a major role in ocular surface morphogenesis and its ablation leads to damage and various eye manifestations at postnatal stages.

MEK kinase 1 (MEKK1) is a member of the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) family that activates MAPKs through the MAPKKK-MAPKK signaling cascade [1]. Among the three major groups of MAP kinases, including the Extracellular signal-Regulated Kinases (ERKs), the c-Jun N-terminal Kinases (JNKs), and p38 [2], MEKK1 preferentially regulates the JNK pathway [3-6]. MEKK1 ablation selectively blocks JNK activation by a subset of physiological and stress stimuli, but has only a marginal or no effect on the activation of ERK and p38 [7,8]. Although MEKK1 has been implicated in diverse and cell type specific biological functions, including apoptosis induced by stress stimuli [9], T cell activation [10], cardiac hypertrophy [11,12], and keratinocyte terminal differentiation [13], its most important in vivo role is the regulation of eye development. MEKK1-deficient mice complete embryonic development and are born with a relatively normal appearance, except for the “eye-open at birth” (EOB) phenotype [14]. Our recent studies show that MEKK1 transduces activin B signals that lead to the movement of eyelid epithelium and eyelid closure [15].

The cornea is a highly specialized transparent tissue consisting of three cell types: The outer epithelium, the inner endothelium, and the intermediate stroma. The corneal stroma is composed of dendritic keratocytes and collagen fibrils associated with proteoglycans in an arrangement that allows corneal transparency and curvature for refractive power [16,17]. The corneal epithelial cells are tightly adhered to one another through specialized intercellular structures, including tight junctions, adherens junction, and desmosomes, responsible for its barrier function against fluid loss, pathogen entrance, and pressure [18,19].

The role of MEKK1 in eyelid development is mediated by JNK, which phosphorylates c-Jun at the N-terminus and enhances its transcriptional activities [15,20,21]. c-Jun is believed to modulate cell behavior such as proliferation, differentiation, and embryonic tissue morphogenesis via controlling gene expression [22]. Its expression in the corneal epithelium during development suggests that c-Jun may play a role in cornea morphogenesis [22,23]. Many genes crucial for corneal epithelium functions have c-Jun and AP-1 binding cis-elements in their promoters [24-26] and the JNK pathway and
c-Jun have been implicated in corneal epithelial differentiation, wound healing, and cell migration [24,27,28].

In this report, we show that MEK kinase 1 is a crucial signaling kinase that regulates eye development and its ablation results in severe eye pathology. The corneal stroma of MEKK1 deficient fetuses, although failing to display an obvious morphological defect during embryonic development, exhibits a marked reduction in extracellular matrix deposition, likely to be the result of decreased extracellular matrix gene transcription. MEKK1 does not control corneal epithelial cell proliferation, differentiation, or the integrity of intercellular structures such as adherens junction and desmosomes; it is, however, required for the formation of tight junctions in the developing corneal epithelium. The detection of c-Jun expression in corneal epithelial cells during mouse embryonic development and the obvious reduction of c-Jun N-terminal phosphorylation in MEKK1-deficient cornea strongly suggest that c-Jun is a target of MEKK1 signaling that controls corneal morphogenesis through modulating c-Jun-dependent gene transcription.

METHODS

Tissue preparation and histological analyses: The generation of wild type and Mekk1<sup>−/−</sup> mice was as described previously [15]. Embryos at different gestation days or adult tissues were fixed in 4% paraformaldehyde, dehydrated with a graded ethanol series and embedded in paraffin. All experiments conducted with these animals have been approved by the University of Cincinnati Animal Care and Use Committee and was in compliance with the Guide for the Care of Laboratory Animal Research. Sections (5 µm) were stained with hematoxylin and eosin according to standard procedures. Sections from postnatal day 27 were processed with periodic acid/Alcian blue/ Schiff’s reagent (PAS) and counterstained with hematoxylin, as described [29]. Masson’s trichrome staining on eye tissues was carried out by the Histological Core at the University of Cincinnati.

In situ hybridization: To identify the cell types that express Keratocan, Lumican, and Collagen I mRNA, in situ hybridization was performed as described previously [30]. In brief, antisense and sense digoxigenin-labeled riboprobes (Roche Applied Science, Indianapolis, IN) of mRNA for Keratocan, Lumican, and Collagen I were synthesized and used for in situ hybridization on paraffin sections (5 µm) mounted on Superfrost/Plus microscope slides (Fisher Scientific, Hampton, NH). To remove nonspecifically bound probes, slides were subjected to a stringent wash with 0.5X SSC at 65 °C and treated with 20 µg/ml RNase (Sigma-Aldrich, St. Louis, MO) at room temperature for 1 h, followed by washing with 0.2X SSC at 65 °C as described previously [30]. The hybridization signals were visualized with anti-digoxigenin antibody-alkaline phosphatase conjugates using procedures recommended by the manufacture.

RNA isolation, reverse transcription, and real-time quantitative PCR: Total RNA was isolated from corneas of wild type and Mekk1<sup>−/−/−</sup> fetuses at stages E15.5 and E16.5 using Tri-reagent (Molecular Research Center Inc., Cincinnati, OH) and purified with the RNAeasy Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription was performed using SuperScript II RNase H-reverse transcriptase (Invitrogen Life Technology, Carlsbad, California). Real-time PCR was carried out with Cepheid PCR Analyzer using SYBR(r) Green I (Invitrogen Life Technology) as the detection format. The reactions were cycled 35 times under the appropriate parameters for the primers and the fluorescence was measured every 15 s at the end of each cycle to construct the amplification curve. All determinations were performed at least in triplicate. The primers used for mouse Collagen I are 5’-CAC CTA TCA CTG CAA GAA CAG and 5’-CGG ACG AAG CAC ATT TGA GG.

Antibodies and immunohistochemistry: For immunohistochemistry, 5 µm thick paraffin sections of mouse eye tissue were deparaffinized by immersing in xylene, rehydrated and immunostained with specific antibodies. For K14 and K12 staining, visualization of secondary antibody immune complexes was performed with 3,3’-diaminobenzidine (DAB). For other antibody stainings, biotin-conjugated secondary antibody were reacted with avidin-biotin-peroxidase complex (ABC) solution (Vector Laboratories Inc., CA) and the immunoreactivity was developed in DAB, enhanced with 3 mg/ml of nickel ammonium sulfate and 0.3 µl/ml of H<sub>2</sub>O<sub>2</sub>. Specimens were counterstained with methyl green or hematoxylin, dehydrated, mounted in balsam, and observed by light microscopy. Antibodies for BrdU and cytokeratin 14 (K14) were from Sigma-Aldrich, for c-Jun and phospho-c-Jun (S63) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for E-cadherin, β-catenin, desmoplakin, and desmoglein were from BD Biosciences (San Diego, CA) and anti-ZO-1 and anti-occludin were from Zymed Laboratories Inc (San Francisco, CA). Anti-cytokeratin 12 (K12) was as described previously [24].

Cell proliferation: In vivo 5-Bromodeoxyuridine (BrdU) labeling was used to study cell proliferation. Female mice on days 15.5 to 18.5 of gestation were injected intraperitoneally with 200 mg/kg BrdU 2 h prior to sacrifice. The embryos were isolated and fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 µm) were deparaffinized and used for detection of BrdU, by treatment with 2 N HCl followed by immunohistochemistry staining with anti-BrdU antibody as described [31]. BrdU positive cells in the cornea were counted under light microscopy and at least four corneas at each gestation day were studied.

Cell apoptosis: Apoptosis in E15.5-E18.5 embryonic tissues was determined by Terminal deoxynucleotidyl transferase-mediated dUTP Nick End labeling (TUNEL) assay. Paraffin sections of embryonic tissues were treated as described before [32] and incubated with terminal deoxynucleotidyl transferase followed by HRP-dUTP (Sigma-Aldrich). The sections were stained with DAB for color reaction and the numbers of TUNEL-positive cells were scored.
RESULTS
MEKK1-deficient mice exhibit eye destruction and pathology:
In our previous work, we showed that MEKK1 was essential for embryonic eyelid closure [15] and that the MEKK1-deficient mice displayed the EOB phenotype with otherwise normal eye morphology. A closed eyelid at birth is believed to provide protection to the ocular surface against environmental insults. A defect in eyelid closure may lead to further damage of eye structures at postnatal developmental stages. To evaluate the impact of MEKK1 ablation on later eye development, we compared the histology of eye tissues of wild type and MEKK1 homozygous mutant (Mekk1\(^{∆KD/∆KD}\) ) mice at various postnatal time periods. As early as day 1 after birth, the eyelids in the mutant mice attached to the cornea and they developed obvious cornea irritation and inflammation (Figure 1A). The Mekk1\(^{∆KD/∆KD}\) mice at older ages developed more

Figure 1. Eye pathology in postnatal stages of MEKK1 mutant mice. Coronal sections of wild type (left panels) and Mekk1\(^{∆KD/∆KD}\) (right panels) mice at postnatal day 1 (A; P1) and day 10 (B; P10) were stained with hematoxylin and eosin and photographed using light microscopy. Note the abnormal eye structures that occur in the Mekk1\(^{∆KD/∆KD}\) as early as day one after birth (P1), including eyelid attachment to the cornea (arrowhead). More severe eye pathologies are associated with the homozygous mutant mice of older ages (P10). C: PAS stained coronal sections of paraffin-embedded eye tissues of wild type (1, 3, and 6) and Mekk1\(^{∆KD/∆KD}\) (2, 4, 5, and 7) mice from postnatal day 27 (P27). Selected areas (3 through 7) are shown at higher magnification. Eye pathologies associated with the homozygous mutants, including smaller lens (1 and 2), impaired cornea morphology (1 through 5), irregular retina (6 and 7), overgrowth of the pigmented cells (3 and 4, white arrows). The mutants also completely lack Meibomian glands (6 and 7, arrowheads) and they show corneal stromal neovascularization (4, star) as well as goblet cell hyperplasia in cornea and conjunctiva (5, 6, and 7, asterisk). severe ocular disorders (Figure 1B,C), with apparent alteration in corneal morphology, irregular retinal structures, and outgrowth of pigment cells. The severity of the symptoms varied among the mutants, some of which showed malformed retina and lens (Figure 1B). All Mekk1-mutant mice lacked Meibomian glands yet they showed goblet cell hyperplasia, likely secondary perturbations resulting from EOB [33-36].

Defects in ECM deposition in the corneal stroma of Mekk1\(^{∆KD/∆KD}\) fetuses: Although the impairment in eyelid closure is the most obvious developmental defect associated with the Mekk1\(^{∆KD/∆KD}\) fetuses [15], other eye developmental processes may also be affected by MEKK1 ablation. The eye tissue most likely to be affected would be the cornea, which during normal development is protected by the closed eyelid forming the conjunctiva sac. Hematoxylin and eosin staining showed that the cornea of Mekk1\(^{∆KD/∆KD}\) fetuses displayed the same morphology and thickness as that of the wild type throughout the entire embryonic developmental stages, except for a slight reduction in intensity of eosin staining in a fashion dependent on the developmental stage. This reduction was hardly detectable in the Mekk1\(^{∆KD/∆KD}\) fetuses prior to E14.5, it began to be observed at E15.5, and became more evident in E16.5 fetuses and older animals (data not shown). As ECM are the major non-cellular constituents of corneal stroma that might make a major contribution to the intensity of eosin staining, the Mekk1\(^{∆KD/∆KD}\) fetuses may suffer a reduction in extracellular matrix deposition in the corneal stroma. To measure directly ECM levels, we performed Masson’s trichrome staining, on the eye tissues of wild type and Mekk1\(^{∆KD/∆KD}\) fetuses at various gestation stages (E14.5-E18.5). While collagen deposition was readily observed in the corneal stroma of E15.5 wild type fetuses, it was hardly detectable in that of Mekk1\(^{∆KD/∆KD}\) fetuses. At later embryonic developmental stages (E16.5-18.5), there was a significant increase in ECM deposition in the corneal stroma of wild type, but much less so in that of Mekk1\(^{∆KD/∆KD}\) fetuses (Figure 2A).

The major component of corneal ECM is collagen I, associated with several proteoglycans, such as keratocan and lumican, to form a well-orchestrated ECM network. To search...
for the particular ECM component that might be down-regulated in the mutant cornea, we carried out in situ hybridization using RNA probes specific for Collagen I, Keratocan, and Lumican. All the ECM genes examined were more abundantly transcribed in the wild type than in the Mekk1\(^{\text{KD/KD}}\) cornea (Figure 2B). To provide a quantitative measure, we evaluated the levels of Collagen I mRNA by reverse transcription and real-time PCR in the developing corneal tissues. MEKK1 ablation caused a reduction in Collagen I transcripts by approximately 50% in corneas from E15.5 and E16.5 fetuses (Figure 2C). Hence, a decrease in transcription of several ECM gene batteries by MEKK1 ablation may contribute to the overall ECM reduction in the corneal stroma.

**MEKK1 ablation does not affect corneal cell proliferation and apoptosis:** Because TGFβ2 signaling was required for keratocyte proliferation and ECM gene expression, TGFβ2 knockout mice had less ECM deposition in corneal stroma [37]. A reduction in keratocytes might also be the reason for the observed ECM reduction in Mekk1\(^{\text{KD/KD}}\) fetuses, because, as MEKK1 has been shown to provide survival signals its deficiency might reduce the keratocyte number by rendering the cells less proliferative or more susceptible to apoptosis [7]. We tested these possibilities by measuring apoptosis using TUNEL assays and cell proliferation by BrdU incorporation on corneas of E13.5 to E18.5 fetuses. The overall numbers of apoptotic cells remained undetectable in all corneal cell types, although evident programmed cell death was observed in the brain of the developmental period examined (data not shown). Most importantly, there was no increase in keratocyte apoptosis in the cornea as a result of MEKK1 ablation. Next, we examined cell proliferation by comparing the rate of BrdU incorporation at different developmental stages (E15.5-E18.5) of wild type and Mekk1\(^{\text{KD/KD}}\) fetuses. Very few, if any, BrdU positive cells were detected in the corneal epithelium, yet no differences in the average numbers of proliferating cells were found between wild type and mutant tissues (Figure 3). Both stromal keratocytes and endothelial cells were clearly more proliferative than epithelial cells, but the proliferation index was not affected by MEKK1 ablation throughout the entire embryonic stages examined. These results suggest that MEKK1 does not affect corneal cell proliferation or apoptosis, therefore the reduced corneal ECM deposition in the mutant fetuses is not caused by a decrease in the numbers of keratocytes. In agreement with this notion, we found that the number of hematoxylin stained nuclei in the corneal stroma was not altered by MEKK1 ablation, with an average of 145-

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**Figure 2.** Extracellular matrices deposition in the developing cornea. 
A: Coronal sections of paraffin-embedded eye tissue of wild type and Mekk1\(^{\text{KD/KD}}\) fetuses at various days of gestation from E14.5-18.5 were analyzed by Masson’s trichrome staining. B: Wild type and Mekk1\(^{\text{KD/KD}}\) fetuses at stages E15.5 and E16.5 were analyzed by in situ hybridization for mRNAs of Keratocan, Lumican, and Collagen I. Although not obvious at E14.5, the mutant fetuses at E15.5-E18.5 display a reduction in the intensity of staining by Masson’s trichrome in the cornea stroma (arrows), wherein the transcripts for Keratocan, Lumican, and Collagen I were also reduced. C: Total RNA isolated from E15.5 and E16.5 fetuses of wild type and Mekk1\(^{\text{KD/KD}}\) corneal tissue was examined by real-time RT-PCR for Collagen I transcripts. The results represent the average of six experiments with enough RNA needed to reach an arbitrary amplification threshold value normalized to Gapdh mRNA in one sample relative to that in the E15.5 wild type cornea.
170 of wild type and 143-167 of Mekk1∆KD/∆KD keratocytes per corneal section at stages E15.5-E18.5 (data not shown).

MEKK1 is essential for c-Jun N-terminal phosphorylation in corneal epithelium: As discussed before, MEKK1 activity is likely to be mediated via JNK and one of the well-defined nuclear effects of JNK is the phosphorylation of the c-Jun transcription factor and potentiation of c-Jun transcriptional activity [38]. To evaluate whether the effect of MEKK1 in cornea development was mediated through c-Jun, we examined c-Jun expression and phosphorylation in the developing cornea. A large number of c-Jun positive cells were detected in the epithelium, but were completely absent in the stroma and endothelium of the developing cornea, in agreement with what was previously reported by Okada et al [23] (Figure 4A). MEKK1 ablation, however, did not cause changes in c-Jun expression, as the same intensity and numbers of c-Jun positive cells were detected in the cornea epithelium of Mekk1∆KD/∆KD and wild type mice. While many cells were positive for phospho-c-Jun in the corneal epithelium of wild type fetuses, none were detected in that of Mekk1∆KD/∆KD (Figure 4B). This finding, together with our previous results in the developing eyelids [15], strongly suggest that c-Jun phosphorylation in epithelial cells is one of the downstream events of MEKK1 signaling in vivo and that MEKK1 regulated c-Jun phosphorylation may contribute to corneal epithelial development and function.

Corneal epithelium differentiation is independent of MEKK1: c-Jun is a component of the AP-1 complex, whose binding sites are present in the promoter region of many epithelial differentiation genes [24]. As c-Jun phosphorylation may have direct effects on the transcriptional activity of AP-1, the Mekk1∆KD/∆KD fetuses with reduced phospho-c-Jun in the corneal epithelium may show differentiation irregularities and a decrease in differentiation gene expression. The eye tissues of wild type and Mekk1∆KD/∆KD fetuses were examined for the

Figure 3. MEKK1 does not affect corneal cell proliferation. Pregnant females of a Mekk1∆KD heterozygote F1 cross were injected with 200 mg/kg BrdU at different gestation days and fetuses from stages E15.5-E18.5 were genotyped and studied for BrdU incorporation. Eye tissues were subjected to immunohistochemistry using anti-BrdU antibodies and counterstained with hematoxylin. The BrdU positive cells were counted in the developing corneal epithelium (A), stroma (B), and endothelium (C). The results represent the average of four independent experiments. The data are expressed as mean±SEM.

Figure 4. c-Jun phosphorylation in corneal epithelium is dependent on MEKK1. Eye sections of wild type and Mekk1∆KD/∆KD fetuses at stages E15.5 and E16.5 were immunostained with anti-c-Jun (A) and anti-phospho-c-Jun (B) antibodies. The corneal epithelium of both wild type and Mekk1∆KD/∆KD fetuses show positive nuclear c-Jun staining, while c-Jun phosphorylation is evident only in that of wild type fetuses.
expression of cytokeratins K14 and K12, as markers for differentiation of basal and cornea specific keratinocytes, respectively. K14 expression was undetectable in the corneal epithelium of E14.5 fetuses (data not shown), but was readily observed in E15.5 to E18.5 fetuses (Figure 5A). There was no noticeable difference, however, on the level of K14 between wild type and Mekk1ΔKD/ΔKD fetuses, suggesting that MEKK1 ablation had no influence on K14 expression and on basal keratinocyte differentiation in cornea epithelium. K12, the corneal specific protein, was also detected in the corneal epithelium, but only in fetuses of gestation day 16.5 and thereafter (Figure 5B). K12 expression was unaffected by MEKK1 ablation, with the same levels of expression in the corneal epithelium of wild type and Mekk1ΔKD/ΔKD fetuses. It is worth noting that K12 expression in the cornea was completely abolished in the mutant mice at day 1 after birth (data not shown), a likely secondary defect caused by impaired eyelid closure which prematurely exposes the cornea to the outside environment causing corneal epithelium disturbance and limbal deficiency. As far as embryonic development is concerned, MEKK1 appears to be dispensable for the differentiation of corneal epithelium.

**MEKK1 is required for the integrity of tight junctions, but not adherens junctions and desmosomes, in the corneal epithelium:** In addition to its differentiation and K12 expression, corneal epithelium also forms specialized intercellular structures, including tight junctions, adherens junction, and desmosome, characteristics of the functional integrity of corneal epithelium. Proteins that are involved in the formation of several of these structures have been shown to be regulated by c-Jun and AP-1 in other systems [25,39]. It is therefore possible that MEKK1 ablation and the subsequent impairment in c-Jun phosphorylation in corneal epithelium may cause the disturbance of epithelial intercellular structures. To test this possibility, eye tissues of wild type and Mekk1ΔKD/ΔKD fetuses at E15.5 and E16.5 were subjected to immunohistochemistry staining for the expression of protein components of the adherens junction. E-cadherin and β-catenin, both belong to the protein complex of adherens junctions, were detected in the corneal epithelium of E15.5 fetuses and their expression increased slightly at E16.5 (Figure 6A,B). The levels of expression and cellular localization of these adherens proteins were unaffected by MEKK1 ablation. Following the same logic, the expression of desmoplakin and desmoglein was examined to investigate the integrity of desmosomes in the corneal epithelium. Both proteins were also detected at the same level and localized to the cell-cell contact in the corneal epithelium of wild type and Mekk1ΔKD/ΔKD fetuses, unaltered by MEKK1 ablation (Figure 6C,D). We also examined the expression in corneal epithelium of two proteins, ZO-1 and occludin, which are associated with tight junctions. Expression of ZO-1 and occludin was clearly detectable between the suprabasal cells of the corneal epithelium at E16.5 and at slightly lower levels at E15.5 of wild type fetuses (Figure 6E,F). Both proteins, however, were completely undetectable in the cornea of the Mekk1ΔKD/ΔKD fetuses at both gestation stages (Figure 6E,F). Collectively, these results suggest that MEKK1 has a role in the regulation of the tight junction, but not of the adherens junction and desmosomes, in cornea epithelium during ocular surface morphogenesis.
DISCUSSION

The work described in this manuscript focuses on establishing an understanding of the role of MEKK1 in mouse cornea development by comparing wild type and \( \text{Mekk1}^{\Delta\Delta\text{KD}} \) fetuses with respect to cellular signaling and molecular aspects of corneal biology. In the corneal epithelium, we show that MEKK1 is required for transmitting signals that lead to c-Jun N-terminal phosphorylation, which occurs in wild type, but not in \( \text{Mekk1}^{\Delta\Delta\text{KD}} \) fetuses. c-Jun is abundantly expressed in the developing corneal epithelium, in agreement with the results of in situ hybridization by Okada et. al. \[23\]. However, its expression levels are not altered by MEKK1 ablation. From these results, we propose that a lack of c-Jun phosphorylation in \( \text{Mekk1}^{\Delta\Delta\text{KD}} \) fetuses may be responsible for corneal defects during ocular surface development as described below.

Many genes involved in corneal epithelium development, including cytokeratin 12 and 14 (important in differentiation) \[24,40\], E-cadherin (important in adherens junctions), \[25\] and desmoglein (important in desmosomes) \[26\] have AP-1 binding sites in their promoters. However, the expression of keratin 12 signifying corneal type epithelial differentiation, adherens junction and desmosomes is unperturbed in the corneal epithelium of the mutant fetuses, which may explain why the \( \text{Mekk1}^{\Delta\Delta\text{KD}} \) fetuses appear to be normal in corneal epithelium morphology. Instead, the mutants are impaired in the formation of tight junctions, as the expression of tight junction components ZO-1 and occludin are both completely undetectable in the corneal epithelium of the \( \text{Mekk1}^{\Delta\Delta\text{KD}} \) fetuses. The tight junction is a unique intercellular structure, formed by several intracellular zona occludens (ZO) proteins and the transmembrane occludins and claudins. This organization provides a continued seal around the epithelial cells and allows the corneal epithelium to form a resistant barrier against free passage of molecules between cells \[41\]. We predict that the corneal epithelium of the mutant fetuses, having impairment in tight junctions, may suffer from lacking a barrier against paracellular flow.

The promoter sequences of occludin and ZO-1 fail to reveal potential AP-1 regulatory elements. It is therefore still unclear whether c-Jun and AP-1 are directly involved in transcription of genes coding for tight junction proteins. Regulation by the MEKK1 pathway may be at the post-translational level, as the formation of the tight junction structure is dependent largely on phosphorylation of its protein components by signaling kinases \[42\]. The stability of occludin is also determined by E3 ubiquitin ligase, itchy, mediated degradation \[43\].

Figure 6. MEKK1 is required for the formation of tight junctions in the corneal epithelium. Embryonic eye sections of wild type and \( \text{Mekk1} \) homozygous mutant mice at E15.5 and E16.5 were studied by immunostaining for adherens junctions with anti-E-cadherin (A) and anti-\( \beta \)-catenin (B), desmosomes with anti-desmoplakin (C) and anti-desmoglein (D), and tight junctions with anti-ZO-1 (E) and anti-occludin (F). MEKK1 ablation caused a disturbance in corneal epithelium of tight junctions but not adherens junctions and desmosomes because the expression of ZO-1 and occludin is detected in the corneal epithelium of wild type fetuses, but not in the mutants. The expression of E-cadherin, \( \beta \)-catenin, desmoplakin, and desmoglein is unaltered by MEKK1 ablation.
and MEKK1 which, functioning as an ubiquitin ligase, may somehow interfere with occludin degradation [44].

It is worth noting that ZO-1 has been reported to associate with both tight junctions and adherens junctions in the corneal epithelium [45]. Lack of ZO-1 expression may cause a defect of adherens junctions as well as tight junctions in the corneal epithelium of the mutant fetuses. The immunostaining in our system, however, cannot distinguish whether the ZO-1 that we observe is in the tight junctions of apical cells or in adherens junctions of basal epithelial cells. Given that the expression and localization of E-cadherin and β-catenin of the adherens junction are unperturbed by MEKK1 ablation, it is likely that MEKK1 regulates specifically the tight junction and has no control over the adherens junction. The ZO-1 molecules localized to the adherens junctions represent a minor fraction of the entire ZO-1 population and may be undetected by our procedure, although they have been observed using more sensitive immunoelectromicroscopy techniques [45].

In addition to its role in the regulation of the tight junction in corneal epithelium, MEKK1 is involved in the morphogenesis of the corneal stroma. The MEKK1 deficient fetsuses show a marked decrease in ECM deposition with reduced transcription for all the ECM components examined. Many genes of the corneal ECM family, such as Collagen 1 and Keratocan [39,46,47], have AP-1 binding sites in their promoters. However, c-Jun expression is undetectable in corneal stroma keratocytes during development. It is therefore unlikely that a c-Jun dependent mechanism is directly responsible for ECM gene transcription in the developing cornea. As discussed before, many epidermal epithelium functions, such as tight junction formation and migration, are compromised in Mekk1 Δ/Δ mice (Figure 6E,F) [15]. Thus it is of interest to speculate that the altered ECM deposition in the mutant mice may be derived from the perturbation of epithelium-mesenchyme interactions. In light of the fact that mice with c-Jun knockout in the epidermis also suffer from reduction in ECM accumulation in corneal stroma (data not shown), we would suggest that lack of c-Jun expression and activity in corneal epithelium might interrupt the generation of paracrine factors that are needed for signaling stromal morphogenesis. The Jun family consists also of Jun B and Jun D, which may exert similar effects as c-Jun, forming an AP-1 complex, on the regulation of gene transcription. The expression patterns of Jun B and Jun D during corneal development, however, have not been studied and their phosphorylation and activities controlled by the MEKK1 pathway have yet to be established. Taken together, our results argue that MEKK1 has a role in the morphogenesis of the corneal stroma by regulating ECM gene transcription, although this function may be secondary to its role in the epithelium.

With both in vitro and in cell culture systems, MEKK1 is believed to play a role in the regulation of cell survival, proliferation, and apoptosis [7]. However, in vivo and in the development of specialized eye tissues, we show that MEKK1 is not a key factor controlling cell proliferation and apoptosis, but rather that it causes decreases in expression of ECM genes in corneal stroma and the complete destruction of tight junctions in corneal epithelium. Some of these effects of MEKK1 are likely to be mediated through c-Jun N-terminal phosphorylation. In addition to its established role in the regulation of eyelid development, MEKK1 appears to also be critical for the development of the cornea. The MEKK1 mediated pathways seem to be important signaling mechanisms in ocular surface morphogenesis and their defects lead to multiple eye perturbations and to pathology that have a severe impact on eye function.

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