Zeb1 Mutant Mice as a Model of Posterior Corneal Dystrophy

Yongqing Liu,[1,2] *Xiaoyan Peng,*[1] *Jinlian Tan,*[3] *Douglas S. Darling,*[3] *Henry J. Kaplan,*[1] *and Douglas C. Dean*[1,2]

Purpose. The zinc finger transcription factor Zeb1 binds to E-box-like sequences and is important for maintaining repression of epithelial specification genes in vivo. Overexpression of Zeb1 in cancer triggers epithelial–mesenchymal transition, which facilitates metastasis. The mutation of *ZEB1* in humans is linked to posterior polymorphous corneal dystrophy (PPCD), in which an epithelial transition of the corneal endothelium is associated with abnormal endothelial proliferation. The purpose of this study is to determine whether *Zeb1* null or heterozygous mice may provide an animal model for PPCD.

Methods. Corneal morphology, protein and mRNA expression, and cell proliferation were compared in wild-type and *Zeb1* gene knockout mice by immunostaining, real-time PCR, and BrdU incorporation. mRNA expression in isolated embryo fibroblasts derived from wild-type, *Zeb1* heterozygous, and null mice was analyzed by real-time PCR

Results. *Zeb1* null mice late in gestation show ectopic expression of epithelial genes in the corneal endothelium and keratocytes, including the basement membrane component COL4A3, which is ectopically expressed by the corneal endothelium in PPCD. These embryos also show abnormal corneal endothelial and keratocyte proliferation, corneal thickening, and corneolenticular and iridocorneal adhesions. Adult *Zeb1* heterozygous mice exhibit these same corneal defects. The ectopic expression of epithelial genes extended to embryonic fibroblasts derived from *Zeb1* heterozygous and null mice, suggesting that Zeb1 may have a more general role in the suppression of an epithelial phenotype.

Conclusions. The authors conclude that *Zeb1* heterozygous and null mice show features of PPCD and thus should provide an animal model for genetic dissection of pathways contributing to the disease. (*Invest Ophthalmol Vis Sci.* 2008;49:1843–1849) DOI:10.1167/iovs.07-0789

From the [1]Department of Ophthalmology and Visual Sciences and the [2]James Graham Brown Cancer Center, University of Louisville Health Sciences Center, Louisville Kentucky; and the [3]Departments of Periodontics, Endodontics, and Dental Hygiene, Center for Oral Health and Systemic Disease, University of Louisville School of Dentistry, Louisville, Kentucky.

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Corresponding author: Douglas C. Dean, Department of Ophthalmology and Visual Sciences, University of Louisville Health Sciences Center, 301 E. Muhammad Ali Boulevard, Louisville, KY 40202; dcdean01@louisville.edu.

Posterior polymorphous corneal dystrophy (PPCD; also known as PPMD) is autosomal dominant and bilateral, and it is characterized by transition of the corneal endothelium to an epithelial phenotype, hyperplasia of the endothelium, disrupted Descemet membrane, opacities, iridocorneal adhesions, corneal edema, corectopia, and secondary glaucoma.[1–6] There appears to be incomplete penetrance in PPCD, leading to a wide spectrum of clinical outcomes in affected families (e.g., all or only a portion of defects are listed here).

Several different genes have been linked to PPCD, leading to subclassification of the disease based on gene mutation. PPCD1 has been reported to occur with mutations of the visual systems homeobox 1 gene (*VSX1*); however, more recent studies appear to exclude the *VSX1* locus.[7–9] PPCD2 appears to occur from a mutation of the *COL8A2* gene, which encodes the α2 chain of type VIII collagen, a major component of Descemet membrane. Thus far, only a single mutation has been identified in two related patients.[10] Gene mutations in the zinc finger homeodomain transcription factor *ZEB1* (also known as *TCF8*) are linked to PPCD3.[6,11] Indeed, in these studies it was estimated that *ZEB1* mutations may be responsible for half of all PPCD cases.[6] Accordingly, a recent study of 10 unrelated Czech and British families found that four of the families carried *ZEB1* mutations.[11] No linkage to either *COL8A2* or *VSX1* was found, suggesting that an unknown mutation is responsible for PPCD in the other six families. Mutations in type IV collagen genes, such as *COL4A3*, *COL4A4*, and *COL4A5*, lead to basement membrane defects and cause the fibrotic hereditary renal disease known as Alport syndrome, which can also be associated with PPCD.[12] COL4A3 expression was found to be deregulated in the cornea in PPCD, and *COL4A3* contains consensus binding sites for ZEB1 in its promoter, leading to a potential scenario in which the mutation of *ZEB1* causes the derepression of *COL4A3* and, in turn, an altered Descemet membrane (which is normally composed primarily of type VIII collagen).[6]

Zeb1 is an E-box binding transcription factor whose function is closely linked to TGF-β signaling. It binds to activated Smads (the downstream TGF-β family signaling molecules), facilitating their assembly into a transcriptionally active complex with the transcriptional coactivator p300, thereby augmenting TGF-β-mediated signaling.[13] In the absence of TGF-β signaling, Zeb1 can interact with the transcriptional repressor CtBP to repress transcription.[14] Overexpression of Zeb1 in cancer triggers epithelial-to-mesenchymal transition (EMT; for a review, see Peinardo et al.[15]), a TGF-β–dependent process that facilitates metastasis.[16,17] This cancer EMT is the result of Zeb1-mediated repression of epithelial specification genes such as E-cadherin and genes involved in polarity and tight junction formation. Conversely, *Zeb1* null mice show an opposite phenotype: expansion of the pattern of epithelial gene expression and loss of mesenchymal gene expression.[18] In addition, *Zeb1* heterozygous mice have diminished TGF-β–dependent signaling leading to defects in smooth muscle differentiation after vascular injury.[19] These properties suggest a model whereby mutation of *ZEB1* may be responsible for

ectopic expression of epithelial genes in corneal endothelium, leading to the epithelialization of the cells in PPCD.

Here, we examined *Zeb1* heterozygous and null mice to determine whether these mutant mice might provide an animal model of PPCD. We found that the cornea became thickened in late-stage null mouse embryos with epithelialization of the endothelium and keratocytes, as evidenced by ectopic expression of epithelial markers such as cytokeratin, E-cadherin, and COL4A3. Iridocorneal and corneolenticular adhesions were present, consistent with dysfunctional corneal endothelium. Additionally, this epithelialization of the corneal endothelium was associated with abnormal proliferation of the cells, as occurs in PPCD.[6,20] We found a similar posterior corneal phenotype in adult heterozygous mice. These results suggest that *Zeb1* mutant mice may provide an important tool for analysis of pathways involved in PPCD.

Materials and Methods

Mouse Cornea and MEF Isolation and Culture

Zeb1 heterozygous mice[21] in a C57BL/6 background were maintained as a colony by breeding with wild-type C57BL/6 mice. Heterozygotes were mated to obtain *Zeb1* null embryos. Mouse genotyping was conducted as described.[21] All animals were handled according to the regulations of the Institutional Animal Care and Use Committee, and all procedures adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Embryonic corneas and fibroblasts (MEFs) were isolated from embryonic day (E) 18.5 and E13.5 mouse embryos, respectively. For MEF isolation, corneal dissection and for Immunostaining below, pregnant mice were exposed to CO_2, and embryos were surgically harvested. A routine MEF isolation procedure was used: the head and visceral organs were removed, and the remaining body cavity was minced and trypsinized. MEFs were cultured in 10% CO_2 at 37°C in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and cells were split 1:3 as they became confluent. For corneal isolation, eyes were removed after embryo harvest, and corneas were dissected from the eyes under a dissecting microscope. Isolated corneas were immediately frozen in liquid nitrogen. Corneas of the same genotype were pooled.

RNA Extraction and Real-Time PCR

Total RNA from corneas or cultured MEFs was isolated using solution (Trizol; Invitrogen, Carlsbad, CA). cDNA was synthesized with an RT kit (Invitrogen) according to the manufacturer's protocol.

With the use of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi), primer sets were designed to generate 100- to 200-base pair PCR products that bridged two separate exons. Primer sequence, melting temperature (Tm), and PCR product sizes are listed in Supplementary Table S1, online at http://www.iovs.org/cgi/content/full/49/5/1843/DC1. Real-time PCR was performed in 25-μL reactions containing 0.25-μL aliquots of cDNA, gene-specific primers, and fluorescent dye (SYBR Green I; Molecular Probes, Eugene, OR), in a PCR system (Mx3000P Real-Time; Stratagene, Cedar Creek, TX). Parameters were set at 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, for a total of not more than 45 cycles. The fluorescent intensity of the dye (SYBR Green; Molecular Probes) was monitored at the end of each extension step; relative amounts of the target cDNA were estimated by the threshold cycle (*Ct*) number and compared with two control genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Three independent samples were analyzed for each condition or cell type, and each sample was compared in at least three independent amplifications.

Immunohistochemistry

Mouse embryos or enucleated adult eyes were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 5 μm. These sections were used for hematoxylin and eosin (H&E) staining or for immunofluorescence. For immunofluorescent staining, the primary antibody dilutions for Zeb1 (raised against the central homeodomain region of the protein expressed in bacteria and then used to immunize rabbits),[22] COL4A3 (mAb 8D1; from Dorin-Bogdan Borza, Vanderbilt University),[23] cytokeratin (Biolegend, San Diego, CA), and E-cadherin (BD PharMingen, San Jose, CA) were 1:100, 1:10, 1:10, and 1:50, respectively, whereas the secondary antibody dilution was 1:300 for both anti-rabbit IgG conjugated with dye (Alexa Fluor-488; Molecular Probes) and anti-mouse IgG conjugated with Cy3 (Sigma, St. Louis, MO). The slides were mounted with coverslips using antifade medium (Permount) and were viewed under a confocal microscope (Carl Zeiss, Oberkochen, Germany). The same exposure time was used to capture images of wild-type and null embryo sections. Images were captured and assembled using Zeiss software (LSM5 Image Examiner). Images were transferred to a PowerPoint file to create the figures, and the PowerPoint file was then converted to a PDF document.

BrdU Incorporation Assays

Two hours before collection of embryos at E13.5 or E15.5, mothers received intraperitoneal injections of 40 mg/kg of 5′-bromodeoxyuridine (BrdU) in PBS. Embryos were fixed, embedded in paraffin, and sectioned at 5 μm. Sections were incubated with 0.1% Tween 20, 4% sheep serum, and 2% bovine serum albumin (BSA) (Sigma) for 1 hour. Primary antibody against BrdU[22] was applied to the sections at 1:50 and incubated at 4°C overnight. Slides were then incubated at 1:300 dilution either with anti-mouse IgG conjugated to Cy3 (Sigma) or with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000) in 1% (vol/vol) normal goat serum in PBS for 1 hour, washed three times for 5 minutes each in PBS, and then developed using DAB peroxidase reagents. The slides were viewed with a confocal microscope (Carl Zeiss), and images were captured and processed as described.

To quantify BrdU incorporation, at least 50 cells were counted for corneal endothelium, corneal epithelium, keratocytes, and lens epithelium, and the percentage of BrdU-positive cells was determined. Littermates were used for these studies. At least two different mice were used for each genotype and age, and five adjacent 5-μm sections were counted for each mouse (see Figs. 5A–C for the regions of the corneas assessed).

Corneal Thickness Measurements

Corneal thickness was measured in H&E-stained sections of the different *Zeb1* genotypes and at different ages. Littermates were used for these studies. At least two different mice were used for each genotype and age, and five adjacent 5-μm sections were counted for each mouse (see Figs. 5A–C for the regions of the corneas measured).

Corneal keratocyte nuclei were counted in adult (4-month-old) wild-type and heterozygous littermates. Three mice were used for each genotype. The same regions used to determine corneal thickness were used to count keratocyte nuclei. Keratocyte nuclei were counted in a 200-μM linear region of the corneas. To do this, a 200-μM square box was placed on the corneal image, and keratocyte nuclei within the box were counted. The edge of the box was placed on the corneal epithelium. As a control, corneal epithelial nuclei were counted in the same box.

Results

Expression of Epithelial Markers in Corneal Endothelium and Keratocytes in *Zeb1* Null Embryos

Zeb1 null mice die at birth because of a failure to initiate respiration[21]; thus, they cannot be analyzed postnatally. Therefore, we began by examining corneas in wild-type and *Zeb1* null mice near the end of gestation. Immunostaining for Zeb1 showed expression in the corneal endothelium and in stromal

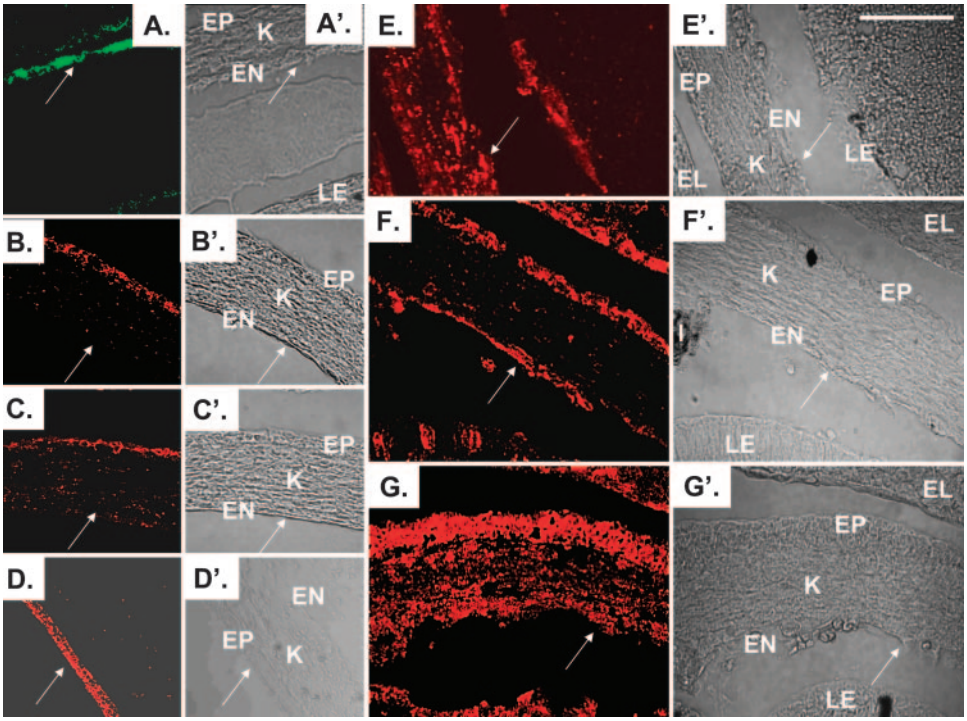

FIGURE 1. E-cadherin becomes ectopically expressed in corneal endothelium, and cytokeratin and COL4A3 become ectopically expressed in corneal endothelium and keratocytes in *Zeb1* null mice. (**A**, **A′**) Immunostaining for Zeb1 is evident in corneal endothelium and keratocytes in wild-type mice. EP, corneal epithelium; EN, corneal endothelium; K, keratocytes; LE, lens epithelium. A Nomarski image is shown on the right in each panel. *Arrows* indicate the same location. (**B**, **B′**) Immunostaining for COL4A3 in the corneal epithelium in wild-type mice. (**C**, **C′**) Immunostaining for E-cadherin in the corneal epithelium in wild-type mice. (**D**, **D′**) Immunostaining for pan cytokeratin in the corneal epithelium in wild-type mice. (**E**, **E′**) Immunostaining for COL4A3 expands to corneal keratocytes and endothelium in *Zeb1* null mice. EL, eyelid. (**F**, **F′**) Immunostaining for E-cadherin expands to the corneal endothelium in *Zeb1* null mice. (**G**, **G′**) Cytokeratin immunostaining expands to corneal keratocytes and endothelium in *Zeb1* null mice. Sections of mice at E16.5 are shown. Scale bar, 100 μm.

keratocytes; no expression was evident in the corneal epithelium (Figs. 1A, 1A′). Epithelial markers including E-cadherin, pan cytokeratin, and COL4A3 were absent from the corneal endothelium and keratocytes but were evident on epithelium of the cornea, lens, and eyelid (as well as the skin; Figs. 1B, 1B′, 1C, 1C′, 1D, 1D′; results not shown).

Next we asked whether there is evidence of an epithelialization of corneal endothelium and keratocytes in *Zeb1* null mice (e.g., do the epithelial markers become ectopically expressed on corneal endothelium and keratocytes in *Zeb1* null mice?). *Zeb1* null littermates at E16.5 were then immunostained for COL4A3, cytokeratin, and E-cadherin. Each of these epithelial markers became ectopically expressed on the corneal endothelium in *Zeb1* null mice, and COL4A3 and cytokeratin were also evident on keratocytes in the null mice (Figs. 1E, 1E′, 1F, 1F′, 1G, 1G′). Although these epithelial markers became ectopically expressed on corneal endothelium and on keratocytes in the null mice, their expression on corneal epithelium or on the epithelium of the lens, eyelid, or skin did not diminish in the null mice. These results indicate that Zeb1 is required to prevent the expression of epithelial specification genes on corneal endothelium and keratocytes, but loss of Zeb1 did not affect expression of these markers on epithelial cells (where Zeb1 was not expressed). As a negative control, immunostaining for each of the proteins was dependent on the primary antibody, and, importantly, we also found that the *Zeb1* null mutation eliminated Zeb1 immunostaining in the eye at E16.5 (results not shown).

COL4A3 and E-cadherin mRNAs Expressed Late in Gestation in the Corneas of *Zeb1* Null Mice But Not of *Zeb1* Heterozygotes

Next we dissected corneas from wild-type, heterozygous, and null littermates at E18.5 and compared the levels of mRNA expression for COL4A1, COL4A3, and E-cadherin (using β-actin and GAPDH mRNAs as controls). As with protein expression, we found significant induction of COL4A3 and E-cadherin mRNAs using real-time PCR in the corneas of *Zeb1* null mice (Fig. 2). There was only a modest increase in COL4A1 mRNA in the null corneas. Little difference in mRNA levels was evident between wild-type and heterozygous corneas at this developmental stage.

Increased Proliferation of the Corneal Endothelium and Keratocytes Late in Gestation in *Zeb1* Null Mice

Epithelialization of the corneal endothelium in PPCD is associated with abnormal cell proliferation. We noticed that corneas from *Zeb1* null mice at E15.5 and E17.5 appeared thicker than their wild-type littermates (Fig. 3A; also shown in Fig. 5), suggesting that the *Zeb1* null corneas may be undergoing abnormal proliferation to add cells to the cornea. To assess

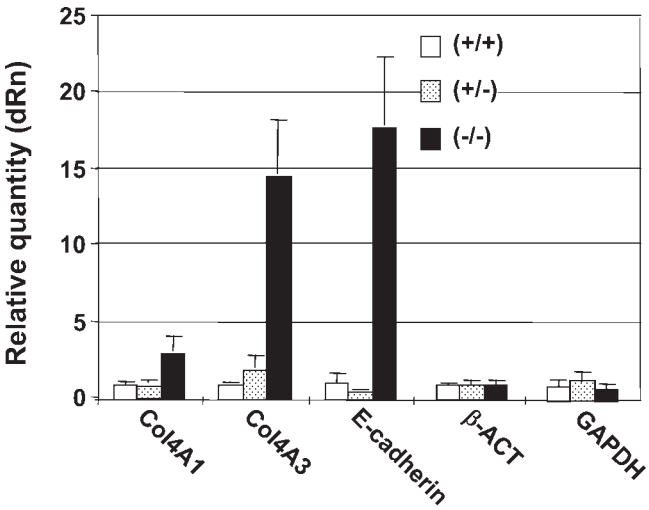

FIGURE 2. Induction of COL4A3 and E-cadherin mRNAs in the cornea in *Zeb1* null mice. mRNA levels in corneas isolated from littermates at E18.5 were quantified by real-time PCR and compared with β-actin and GAPDH mRNAs as a control. Corneas from three embryos were pooled for each genotype.

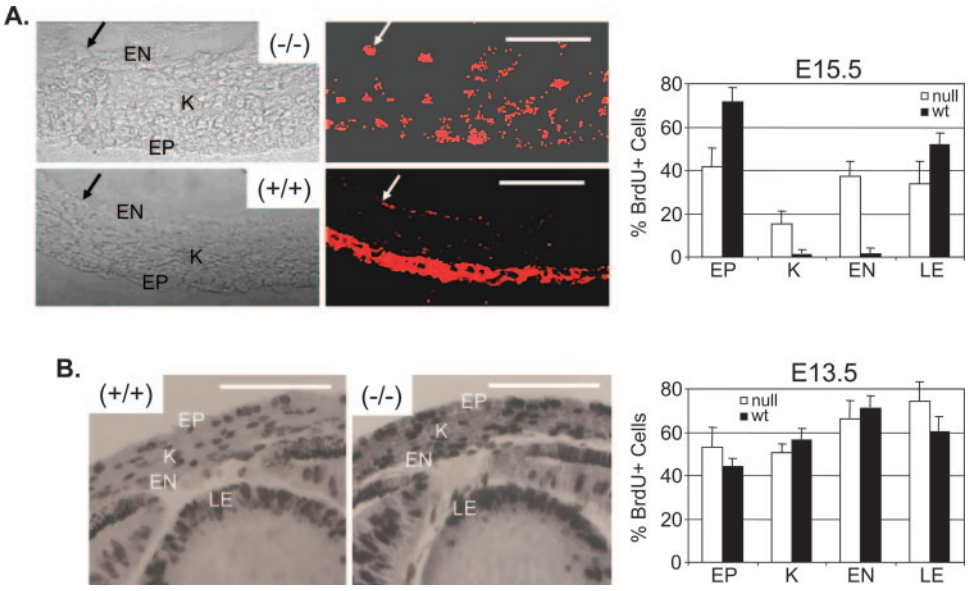

FIGURE 3. Abnormal corneal proliferation occurs in *Zeb1* null mice at E15.5 but not at E13.5. Mice at E13.5 or E15.5 were injected with BrdU, and embryos were harvested 2 hours later and sectioned for immunostaining with anti-BrdU antibody. (**A**) Immunofluorescent staining for BrdU and quantification of BrdU incorporation in corneal epithelium (EP), endothelium (EN), keratocytes (K), and lens epithelium (LE) is shown at E15.5. *Arrows* indicate the same location in the images. Nomarski images are shown at the *left* in each panel. (**B**) Immunoperoxidase staining for BrdU and quantification of BrdU incorporation into the cornea at E13.5. Scale bars, 100 μm. Corneal areas counted in (**A**) and (**B**) are denoted by the asterisks in Figures 5A and 5C. Error bars represent SD. Littermates were examined, at least two different mice were examined for each genotype, and five adjacent 5-μm sections were counted for each mouse.

proliferation in the cornea, pregnant mice at E13.5 and E15.5 were injected with BrdU 2 hours before embryos were harvested. BrdU incorporation, as a measure of cell proliferation, was then analyzed by immunostaining in sections of wild-type and null littermates. Proliferation was evident throughout the corneas in wild-type and *Zeb1* null mice at E13.5 (Fig. 3B). However, proliferation became largely restricted to the epithelium in wild-type corneas by E15.5 (Fig. 3A). Although the corneal epithelium and the lens epithelium remained proliferative in the null mice at E15.5, endothelial cells and keratocytes were also proliferative in the null corneas (Fig. 3A). Thus, ectopic expression of epithelial markers on the corneal endothelium and keratocytes in *Zeb1* null mice is associated with abnormal proliferation of these cells at E15.5.

Increased Corneal Thickness and Corneolenticular, Iridocorneal, and Iridolenticular Adhesions Late in Gestation in *Zeb1* Null Mice

Consistent with increased proliferation in the corneas of *Zeb1* null late-stage embryos, corneas from *Zeb1* null mice at E17.5 mice were thicker than corneas from their wild-type littermates, due at least in part to an increase in the number of keratocytes (Figs. 4A, 4B). Corneal thickness in heterozygous mice was not statistically different from that in wild-type mice. By contrast, corneas from wild-type and *Zeb1* null mice at E13.5 (in which corneal proliferation was similar; Fig. 3B) showed similar thicknesses (Fig. 4A).

In addition to increased corneal thickness, iridocorneal, corneolenticular, and iridolenticular adhesions were evident in *Zeb1* null mice at E17.5 (Figs. 5A–F). These corneolenticular adhesions frequently led to corneal disruption, with the endothelium torn away from the cornea and adherent to the lens epithelium (Fig. 5E). Iridocorneal adhesion led to loss of the iridocorneal angle in the null embryos (Figs. 5C–D). Such pathologic defects are indicative of defective corneal endothelium and are associated with posterior corneal dystrophies such as PPCD.

Corneal Thickening and Iridocorneal and Corneolenticular Adhesions in Adult *Zeb1* Heterozygous Mice

We did not detect obvious corneal defects in late-stage *Zeb1* heterozygous embryos, despite the fact that a heterozygous

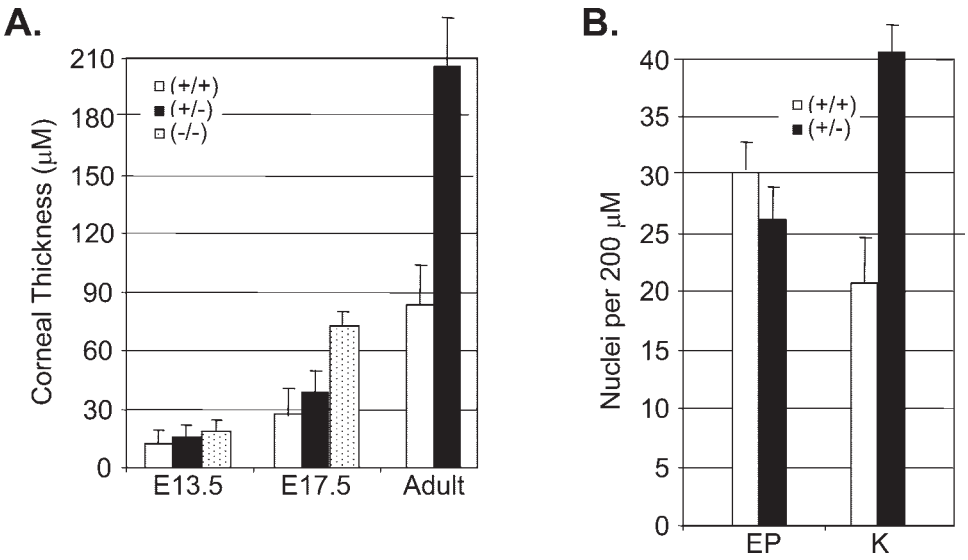

FIGURE 4. Corneal thickening occurs in *Zeb1* null mice at E17.5 and in adult heterozygotes (but not in *Zeb1* null mice at E13.5), and increased keratocyte numbers are seen in heterozygous adults. (**A**). Corneal thickness was assessed in the regions indicated in Figures 5A and 5C. Corneal thickness was measured at E13.5 and E17.5 and in 4-month-old adults. (**B**). Keratocyte nuclei in a 200-μM length of cornea (in areas used to measure corneal thickness in **A**) were counted. Error bars represent SD. Littermates were examined for these measurements, at least two different mice were examined for each genotype, and five adjacent 5-μm sections were analyzed for each mouse.

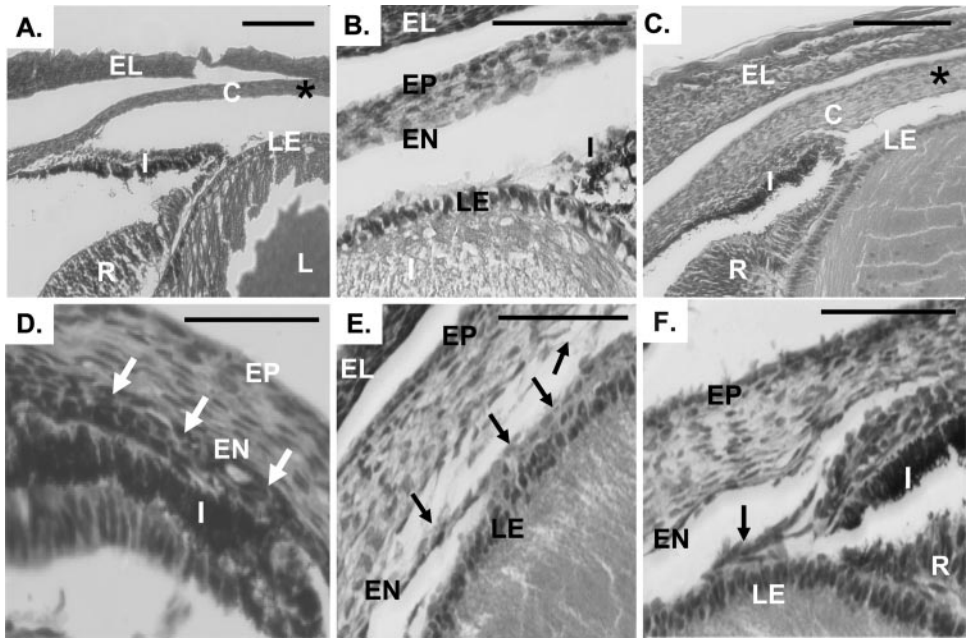

FIGURE 5. Iridocorneal, corneolenticular, and iridolenticular adhesions in *Zeb1* null mice. H&E-stained sections of eyes from wild-type (**A**, **B**) and *Zeb1* null (**C**–**F**) mice. C, cornea; EL, eyelid; I, iris; LE, lens epithelium; R, retina; EN, corneal endothelium; EP, corneal epithelium. (**A**, **C**) *Asterisk* indicates the region of the cornea analyzed for BrdU incorporation (Fig. 3) and corneal thickness (Fig. 4). (**D**) *Arrows* indicate iridocorneal adhesion. (**E**, **F**) *Arrows* show corneolenticular and iridolenticular adhesions, respectively. Scale bars, 100 μm. Sections at E17.5 are shown.

ZEB1 mutation is linked to human PPCD. However, corneal defects in the null mice only became evident late in embryogenesis (implying an age-dependent effect). In humans, PPCD is frequently not diagnosed until 20 to 30 years of age.[20] Therefore, we examined adult (4-month-old) *Zeb1* heterozygous mice for corneal defects. Corneal thickening and increased keratocyte number were evident in the heterozygous mice compared to wild-type littermates (Figs. 4A, 4B). The relative increase in corneal thickness in the adult heterozygotes was similar to that observed in late-stage null embryos.

As with late-stage *Zeb1* null embryos, adult *Zeb1* heterozygotes had iridocorneal and corneolenticular adhesions (Figs. 6A, 6A′, 6B, 6B′). In addition, as with *Zeb1* null embryos, these iridocorneal adhesions led to loss of the iridocorneal angle (Fig. 7).

COL4A3 mRNA Induction in a *Zeb1* Gene Dosage-Dependent Fashion in Embryo Fibroblasts

Zeb1 is crucial for suppressing an epithelial phenotype in mesenchymal and neuroectodermal cells in vivo.[18] Indeed, we have found that E-cadherin becomes expressed in a *Zeb1* dosage-dependent fashion in embryonic fibroblasts[18] (Fig. 8), and this induction of E-cadherin is associated with transition of the *Zeb1* null embryonic fibroblasts to epithelial morphology.[18] Therefore, we wondered whether *Zeb1* gene mutation might also lead to induction of COL4A3 mRNA (*COL4A3* is proposed to be a target of Zeb1 repression whose ectopic expression in corneal endothelium and keratocytes is important in PPCD.[6] Fibroblasts were isolated from wild-type, heterozygous, and null littermate embryos. We found using real-

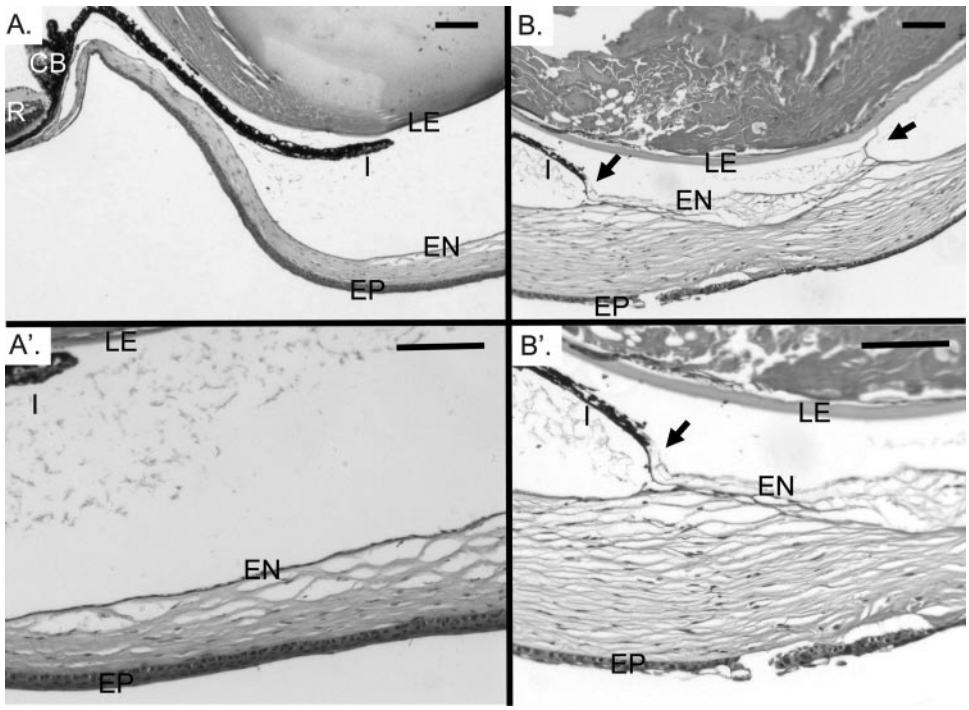

FIGURE 6. Iridocorneal and corneolenticular adhesions in adult *Zeb1* heterozygous mice. H&E-stained sections eyes from adult wild-type (**A**, **A′**) and *Zeb1* heterozygous (**B**, **B′**) mice. *Arrows* in (**B**, **B′**) show corneolenticular and iridocorneal adhesions. EP, corneal epithelium; EN, corneal endothelium, LE, lens epithelium; I, iris; R, retina; CB, ciliary body; R, retina. Scale bars, 100 μm.

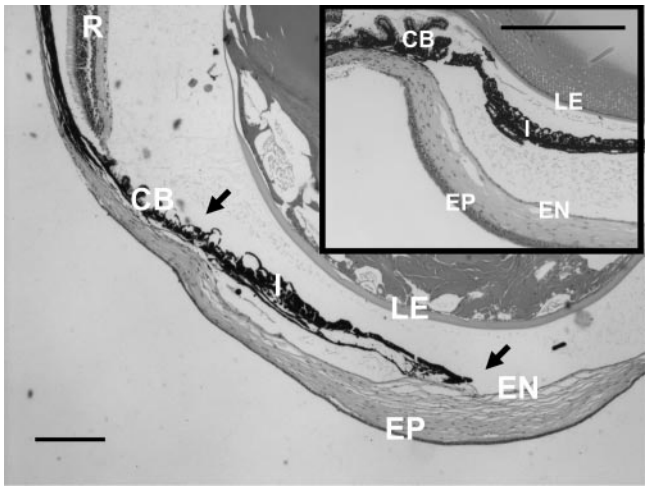

FIGURE 7. Iridocorneal adhesion leads to loss of the iridocorneal angle in adult *Zeb1* heterozygous mice. H&E section through the eye of an adult *Zeb1* heterozygous mouse showing iridocorneal adhesion and loss of the iridocorneal angle. *Inset*: iridocorneal angle in a wild-type littermate. EP, corneal epithelium; EN, corneal endothelium; LE, lens epithelium; I, iris; CB, ciliary body; R, retina. *Arrows*: iridocorneal adhesions. Scale bars, 200 μm.

time PCR that COL4A3 mRNA was indeed induced in heterozygous cells, and there was further induction in the null cells (Fig. 8). There was no change in the expression of β-actin or GAPDH mRNAs, the controls, and relatively little change was evident in COL4A1 mRNA. Contrary to COL4A3 expression, COL4A1 expression is not induced in the corneal endothelium or keratocytes in PPCD.[6]

DISCUSSION

The overexpression of ZEB1 in cancer triggers EMT, which facilitates metastasis in late-stage carcinomas. By contrast, the opposite phenotype (derepression of epithelial specification genes in mesenchymal, neuroectodermal, and endothelial cells) is seen in *Zeb1* null mice, as shown in this study and previously.[18]

Zeb1 shares E-box binding sites on target gene promoters with the Snail family of transcription factors.[24] As with Zeb1, Snail can be overexpressed in cancer, leading to EMT.[15,25] *Snail1* null mice also show ectopic expression of epithelial specification genes, but, as opposed to what occurs in *Zeb1* null mice, this ectopic expression is seen early in embryogenesis (e.g., these defects prevent the formation of the ectodermal–mesodermal boundary required for gastrulation[26]). In *Drosophila*, Snail is responsible for repressing the Zeb1 homologue (zfh1) and preventing its expression until later stages of gestation.[27] Zeb1 expression also follows that of Snail in mouse embryogenesis, and defects in *Zeb1* null mice are not evident until later in gestation.[21] Therefore, Snails may be functioning to suppress epithelial gene transcription early in gestation, whereas Zeb1 assumes this role later in gestation.

Corneal endothelium cells and stromal keratocytes are derived from cranial neural crest, as are many of the mesenchymal cells responsible for craniofacial development (craniofacial defects are prominent in *Zeb1* null mice[21]). Zeb1 is expressed on migrating cranial neural crest,[22] and our results imply that it may be important in regulating the gene expression pattern of these cells as they undergo differentiation. Further, gene expression is also altered in fibroblasts cultured from mutant embryos (an epithelialization of the cells appears to occur) and in neuroepithelial cells in vivo,[18] suggesting that this Zeb1 regulation extends beyond cranial neural crest-derived cells.

Interestingly, the proliferative phenotype seen here in corneal endothelial cells and keratocytes is distinct from the proliferative defects we observed at sites of developing cartilage and in the CNS in *Zeb1* mutant embryos (also sites of developmental defects in the null mice).[18,21] The reason for these opposing effects on proliferation is unclear. However, cell differentiation, survival, and proliferation are closely linked to signals transmitted to the cell through adhesion receptors, such as the integrin family, whose members bind differentially to various extracellular matrix components (for a review, see Lee and Juliano[28]). Descemet membrane in the posterior cornea is a specialized basement membrane composed of a high percentage of type VIII collagen. However, COL4A3 becomes ectopically expressed in corneal endothelial cells and keratocytes in *Zeb1* null mice, thus potentially altering the composition of this crucial basement membrane. Alterations in extracellular matrix composition classically lead to apoptosis or phenotypic changes in cells bound to the matrices. For example, de-differentiating EMT occurs in the renal tubular epithelium, resulting in fibrosis in Alport syndrome (where COL4A3 expression is altered, causing a defective tubular basement membrane; for a review, see Hudson et al.[29]). Normal devel-

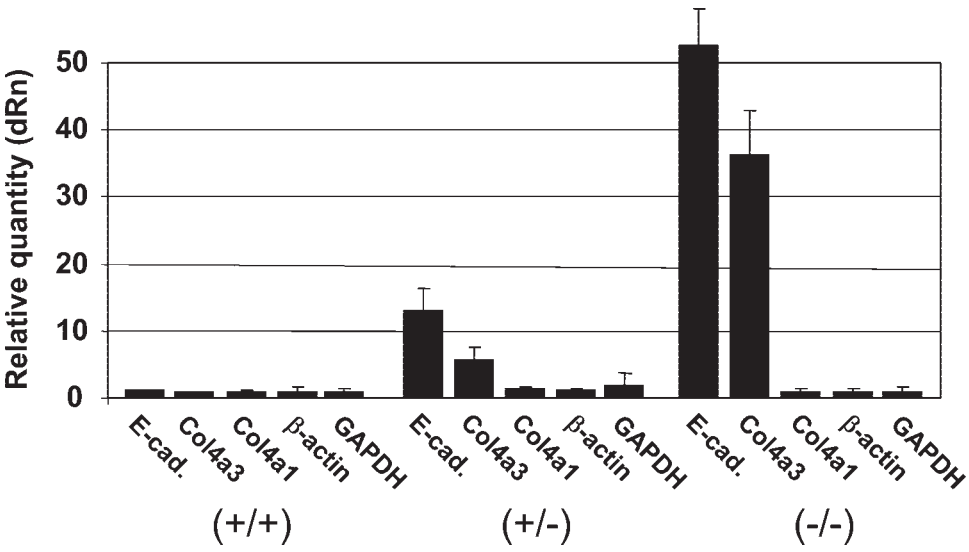

FIGURE 8. Induction of epithelial genes in embryo fibroblasts derived from wild-type, *Zeb1* heterozygous, and *Zeb1* null littermates. Real-time PCR analysis of mRNA levels is shown.

opment of the renal tubular epithelium requires a mesenchymal–epithelial transition during development.[30] Taken together, these results suggest that epithelial–mesenchymal balance is crucial for the formation and maintenance of the tubular epithelium, and this balance appears to be dependent on extracellular matrix composition. It is then of note that PPCD has been reported in patients with Alport syndrome,[12] and it appears that Zeb1 function in the epithelial–mesenchymal/endothelial balance is not confined to the corneal endothelium. Thus, it will be interesting to determine whether there are renal defects in *Zeb1* null mice and whether patients with apparent links between Alport syndrome and PPCD have mutations in *ZEB1*.

Finally, Zeb1 function in vivo is linked to TGF-β signaling; it binds to activated Smads, facilitating their assembly into a transcriptionally active complex with p300.[13] Heterozygous mutation of *Zeb1* leads to defective TGF-β–dependent smooth muscle gene expression and smooth cell differentiation in vivo.[19] Classically, epithelial versus mesenchymal balance in cancer and in normal development is dependent on signaling through the TGF-β family of proteins (e.g., TGF-β drives EMT).[16,17] TGF-β signaling also establishes and maintains cell cycle arrest in differentiating cells through the induction of cyclin-dependent kinases.[31] Both the epithelial phenotype and the abnormal proliferation seen in the corneal endothelium and in the keratocytes of *Zeb1* null mice may prove to be associated with defective TGF-β signaling in the absence of Zeb1. Numerous mouse mutations in genes in the TGF-β family signaling pathway and other pathways are available for crossing with the *Zeb1* heterozygous and null mice. Thus, *Zeb1* mutant mice may provide a model to genetically dissect the pathway(s) affected in posterior corneal dystrophy.

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