Zeb1 Mutant Mice as a Model of Posterior Corneal Dystrophy

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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

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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. 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 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. 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 mothers were exposed to CO2, 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% heat-inactivated fetal bovine serum, and cells were sectioned at 5°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-μM aliquots of cDNA, gene-specific primers, and fluorescent dye (SYBR Green; Molecular Probes) and anti-mouse 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-bromo-2’-deoxyuridine (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 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 cornea. 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; 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
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 littersmates 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 littersmates 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](http://tvst.arvojournals.org/)
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 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
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. 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. Indeed, we have found that E-cadherin becomes expressed in a Zeb1 dosage-dependent fashion in embryonic fibroblasts (Fig. 8), and this induction of E-cadherin is associated with transition of the Zeb1 null embryonic fibroblasts to epithelial morphology. 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). Fibroblasts were isolated from wild-type, heterozygous, and null littermate embryos. We found using real-
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 \( \beta \)-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.

**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.

Zeb1 shares E-box binding sites on target gene promoters with the Snail family of transcription factors. As with Zeb1, Snail can be overexpressed in cancer, leading to EMT. 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). In *Drosophila*, Snail is responsible for repressing the Zeb1 homologue (zfh1) and preventing its expression until later stages of gestation. Zeb1 expression also follows that of Snail in mouse embryogenesis, and defects in Zeb1 null mice are not evident until later in gestation. 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). Zeb1 is expressed on migrating cranial neural crest, 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, 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). 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). 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.). Normal devel-
opment of the renal tubular epithelium requires a mesenchymal-epithelial transition during development.\textsuperscript{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,\textsuperscript{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-\(\beta\) signaling: it binds to activated Smads, facilitating their assembly into a transcriptionally active complex with p300.\textsuperscript{13} Heterozygous mice for Zeb1 have defective TGF-\(\beta\)-dependent smooth muscle gene expression and smooth cell differentiation in vivo.\textsuperscript{19} Classically, epithelial versus mesenchymal balance in cancer and in normal development is dependent on signaling through the TGF-\(\beta\) family of proteins (e.g., TGF-\(\beta\) drives EMT).\textsuperscript{16,17} TGF-\(\beta\) signaling also establishes and maintains cell cycle arrest in differentiating cells through the induction of cyclin-dependent kinases.\textsuperscript{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-\(\beta\) signaling in the absence of Zeb1. Numerous mouse mutations in genes in the TGF-\(\beta\) 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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**References**