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Transcription factor TFAP2B up-regulates human corneal endothelial cell–specific genes during corneal development and maintenance

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The corneal endothelium, which originates from the neural crest via the periocular mesenchyme (PM), is crucial for maintaining corneal transparency. The development of corneal endothelial cells (CECs) from the neural crest is accompanied by the expression of several transcription factors, but the contribution of some of these transcriptional regulators to CEC development is incompletely understood. Here, we focused on activating enhancer-binding protein 2 (TFAP2, AP-2), a neural crest–expressed transcription factor. Using semiquantitative/quantitative RT-PCR and reporter gene and biochemical assays, we found that, within the AP-2 family, the *TFAP2B* gene is the only one expressed in human CECs *in vivo* and that its expression is strongly localized to the peripheral region of the corneal endothelium. Furthermore, the TFAP2B protein was expressed both *in vivo* and in cultured CECs. During mouse development, TFAP2B expression began in the PM at embryonic day 11.5 and then in CECs during adulthood. siRNA-mediated knockdown of TFAP2B in CECs decreased the expression of the corneal endothelium–specific proteins type VIII collagen α2 (COL8A2) and zona pellucida glycoprotein 4 (ZP4) and suppressed cell proliferation. Of note, we also found that TFAP2B binds to the promoter of the *COL8A2* and *ZP4* genes. Furthermore, CECs that highly expressed ZP4 also highly expressed both TFAP2B and COL8A2 and showed high cell proliferation. These findings suggest that TFAP2B transcriptionally regulates CEC-specific genes and therefore may be an important transcriptional regulator of corneal endothelial development and homeostasis.

The cornea tissue consists of three layers: corneal epithelium, corneal stroma, and corneal endothelium. The corneal endothelium is composed of a monolayer and the Descemet's membrane (1). The two major functions of the corneal endothelium are pumping out interstitial fluid from the corneal stroma and serving as a barrier for the stroma to prevent the entry of aqueous humor into the corneal stroma, which eventually prevents the thickening of the cornea (2).

Human corneal endothelial cells (CECs)[4] are differentiated from the neural crest via the facial neural crest and periocular mesenchyme (PM) (3). The original neural crest cells express specific differentiation markers, including p75 neurotrophin receptor (*p75*), SRY-box 9 (*SOX9*), *SOX10*, *Snail*, and *Slug*. The cranial neural crest, a lineage of the neural crest, gives rise to facial bones, cartilage, peripheral neurons, glia, and parts of the eye (4). Around the region of the developing eye, the PM migrates into the space between the surface ectoderm and lens vesicle and then differentiates into the corneal stroma and corneal endothelium. The PM expresses two important transcription factors: paired-like homeodomain 2 (*PITX2*) and forkhead box C1 (*FOXC1*) (5, 6). Mutations in these genes may lead to Axenfeld–Rieger syndrome (7, 8), which manifests as abnormalities of the anterior segment of the eye, including the cornea. Previously, we reported that human corneal endothelial progenitor cells retain some properties of both the neural crest and PM, including the expression of p75, SOX9, PITX2, and TFAP2B (9). Most of these are transcriptional factors and thus may regulate some genes specific to CECs, but their detailed contribution to the development of CECs remains unclear. The

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This article contains Tables S1 and S2.

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[4] The abbreviations used are: CEC, corneal endothelial cell; COL8A1, COL8A2, type VIII collagen α1, α2; FBS, fetal bovine serum; FOXC1, FOXC2, forkhead box C1, C2; p75, p75 neurotrophin receptor; PITX2, paired-like homeodomain 2; PM, periocular mesenchyme; SOX9, SOX10, sex-determining region Y (SRY)-box 9, 10; TFAP2, AP-2, transcription factor activating enhancer-binding protein 2; ZO-1, zonula occludens 1; ZP4, zona pellucida glycoprotein 4; E, embryonic day; EMSA, electrophoretic mobility shift assay; TBS, Tris-buffered saline.

transcription factor activating enhancer-binding protein 2 (TFAP2, AP-2) family is expressed in the neural crest and consists of five molecules: TFAP2A, TFAP2B, TFAP2C, TFAP2D, and TFAP2E (10). The TFAP2 family members have a similar amino acid sequence and play an important role in various developmental stages. TFAP2A, TFAP2B, and TFAP2C are important in neural crest and facial formation. TFAP2D is highly expressed in the heart and retina, and TFAP2E is essential for differentiation and maturation of the olfactory bulb (10, 11). The TFAP2 family proteins form a dimer via a helix-span-helix motif and bind to DNA, which is thought to be composed of homodimers or heterodimers (10). Joyce *et al.* (12) reported that TFAP2B expression is higher in CECs than in umbilical cord blood mesenchymal stem cells. Recently, we found that TFAP2B is particularly highly expressed in the corneal endothelium (13). Several analyses of the human CEC transcriptome using next-generation sequencing have also been reported (13, 14). In one of these studies, RNA-Seq data showed only TFAP2B to be consistently expressed, and TFAP2A, TFAP2C, and TFAP2D were rarely expressed in adult and fetal CECs. Expression of TFAP2B was higher than the expression of other corneal endothelium–related transcription factors such as ZEB1 (13).

Regarding their use in regenerative medicine, cultured CECs have limited proliferative ability. Recently, several groups reported that CECs could be generated from multipotent stem cells and somatic stem cells (15–19), but only a few studies have demonstrated the purity of CECs. In our previous study, we found that the ZP4 molecule was a novel CEC-specific marker and was expressed in both *in vivo* and *in vitro* cultured CECs (20). However, to the best of our knowledge, there are few reports on specific transcription factors regulating CEC-specific functions, which are presumably controlled by several CEC-specific genes.

The transcriptional regulation mechanism of TFAP2B in other tissues is not clear, and it is important to explore how it relates to the physiological function of the corneal endothelium. In this study, we examined the transcriptional regulation mechanism of the *TFAP2B* gene, which may lead to the elucidation of differentiation mechanisms important for the study of cell-based therapy in corneal endothelial regeneration.

Results

TFAP2B is expressed in the human CECs

The expression pattern of the AP-2 family in the human corneal endothelium was confirmed by RT-PCR. TFAP2B mRNA was detected, whereas the mRNAs of other family members, TFAP2A, TFAP2C, TFAP2D, and TFAP2E, were not detected (Fig. 1*A*). TFAP2B protein was expressed in human CECs *in vivo* (Fig. 1*B*) with varying expression levels; the expression level in the peripheral region was 2.5-fold greater than that in the central region (Fig. 1*C*), and cells highly expressing TFAP2B were localized in the vicinity of the transition zone. Additionally, the TFAP2B protein was also expressed in *in vitro* cultured CECs (Fig. 1*D*).

TFAP2B is homeostatically expressed during mouse corneal endothelial development

It has been reported that TFAP2B is important for mouse corneal endothelial development, particularly at E11.5 (when the PM migrates between the lens and corneal epithelium) and at E15.5 (when the corneal endothelium forms a single cell layer) (21). To investigate the distribution of TFAP2B during development, we performed immunofluorescence studies using both mouse embryos and adult mice. At E11.5, TFAP2B-expressing cells were localized in the PM (Fig. 2, *A–D*), whereas at E15.5 (Fig. 2, *E–H*) and adulthood (Fig. 2, *I–L*), TFAP2B was expressed in mouse CECs. These results suggested that TFAP2B protein was expressed in the PM and during subsequent steps and that it might be involved in gene regulation during CEC differentiation.

TFAP2B regulates the CEC-specific genes COL8A2 and ZP4

TFAP2B was expressed in mouse CECs throughout all developing stages, from E11 to adulthood, possibly indicating that TFAP2B protein controls the expression of key molecules in CEC development and homeostasis. Then, to test whether genes are regulated by TFAP2B in cultured human CECs, we first analyzed its contribution using siRNA-mediated knockdown. The expression patterns of the neural crest marker SOX9; periocular mesenchyme markers PITX2, FOXC1, and FOXC2; and CEC markers ZO-1, Na^+/K^+-ATPase, COL8A1, COL8A2, and ZP4 were investigated using qRT-PCR in siRNA-treated CECs. Interestingly, the expression levels of COL8A2 and ZP4 mRNA in TFAP2B siRNA–transfected cells were significantly lower than those in the control siRNA–transfected cells (Fig. 3*A*). Furthermore, we validated their protein expression levels by Western blotting (Fig. 3*B*). COL8A2, which is the main component of the Descemet's membrane synthesized from CECs, and ZP4, which is a plasma membranous protein, were selectively expressed *in vivo* in the corneal endothelium (Fig. 3*C*). In a whole-mount immunofluorescence study of the human corneal endothelium tissue, we found that ZP4 protein was strongly expressed in cells highly expressing TFAP2B (Fig. 3*D*) compared with those weakly expressing TFAP2B. In the cell proliferation assay, the proliferative capacity of TFAP2B siRNA–treated CECs also decreased compared with that of control siRNA–treated CECs (Fig. 3*E*). These data suggest that TFAP2B regulates the corneal endothelial cell–specific markers COL8A2 and ZP4 and promotes cell proliferation.

TFAP2B directly regulates transcription of the human COL8A2 and ZP4 genes

Next, we determined the effect of TFAP2B on transcriptional activity in the *COL8A2* and *ZP4* promoters. A luciferase reporter assay was performed to identify the TFAP2B-binding motif in cultured CECs. In previous reports, a sequence consisting of nine nucleotides, 5′-S(G/C)CCTSR(A/G)GGS-3′, was reported to be a common binding sequence of the AP-2 family genes (22, 23). This AP-2–binding consensus sequence was found in the upstream region of the human *COL8A2* and *ZP4* promoters, both at approximately −3.0 kbp from their respective transcriptional start sites (Fig. 4, *A* and *E*). First, we performed EMSA to determine whether TFAP2B binds to the

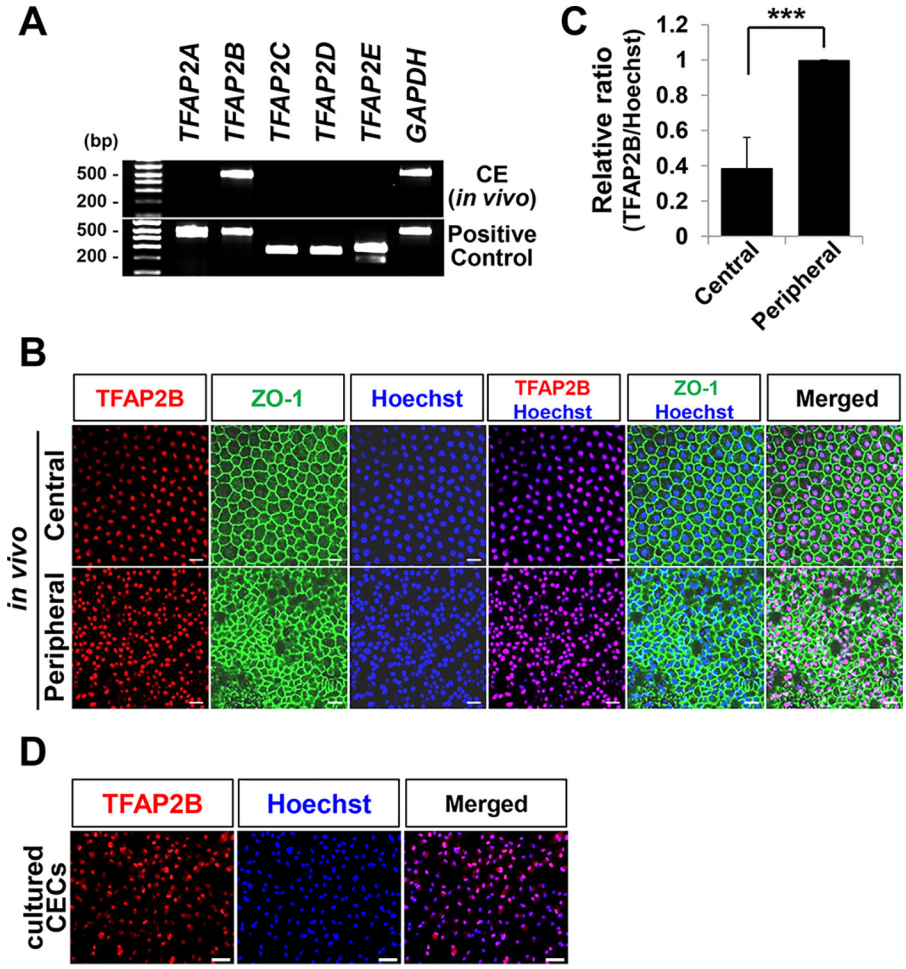

Figure 1. Expression pattern of TFAP2B in human CECs. *A*, gel electrophoresis images of RT-PCR of the TFAP2 family in the human corneal endothelium (*CE*) (*upper*) and positive control (*lower*). cDNA from human neural retina was used as a positive control for RT-PCR. *B*, immunofluorescence of TFAP2B (*red*) and Hoechst 33342 (*blue*) in the corneal endothelium. *Green* signals represent the expression of the ZO-1 protein at CEC junctions. *C*, relative ratio of the fluorescence intensity of TFAP2B *in vivo* between the central and peripheral regions of the corneal endothelium. The fluorescence intensity ratio was calculated from the images of TFAP2B and Hoechst, and the relative ratio between the peripheral and the central regions was determined. The data are shown as the mean ± S.D. (*error bars*) ($n = 6$). ***,*s* $p < 0.001$. *Scale bars*, 20 μm. *D*, immunofluorescence images of TFAP2B (*red*) and Hoechst 33342 (*blue*) in cultured human CECs.

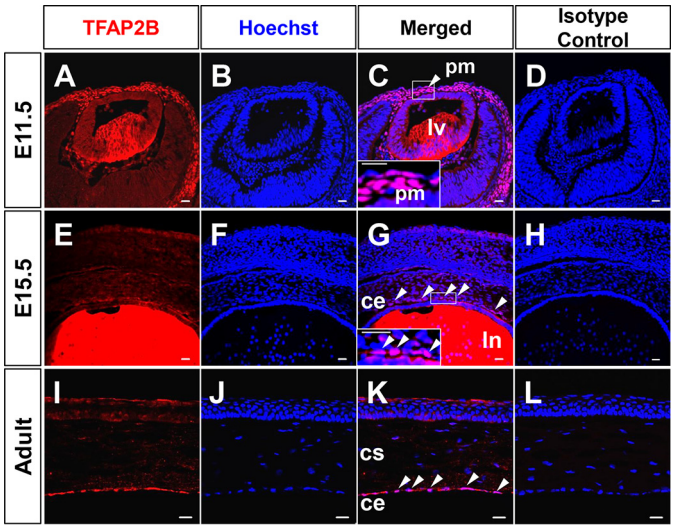

Figure 2. TFAP2B expression in mouse CECs during developmental stages. The photographs show TFAP2B expression (*red*) in embryos and adult mice by immunohistochemistry. *A–D*, E11.5; *E–H*, E15.5; *I–L*, adult mouse. *Arrowheads* indicate mouse CECs. Hoechst 33342 (*blue*) was used to stain the nucleus. *Scale bars*, 20 μm. *pm*, periocular mesenchyme; *lv*, lens vehicle; *ln*, lens; *cs*, corneal stroma; *ce*, corneal endothelium.

sequences of *COL8A2* and *ZP4*. The results showed that the shifted bands were located in the WT sequences, but the artificially introduced mutation sequences did not bind to TFAP2B proteins (Fig. 4, *B* and *F*). The luciferase activity was found to be markedly high in the promoter region–containing vectors compared with that in the basic control vector (Fig. 4, *C* and *G*). The artificially introduced mutation within the TFAP2B candidate binding sites in the *COL8A2* and *ZP4* promoter regions significantly decreased luciferase activity in TFAP2B-overexpressing 293T cells (Fig. 4, *B* and *E*). For each gene, we also performed ChIP-PCR with anti-TFAP2B antibody in CECs. The assay confirmed the binding of TFAP2B proteins to the candidate binding site within the promoters of the *COL8A2* and *ZP4* genes in the primary CECs (Fig. 4, *D* and *H*). These data indicate that TFAP2B protein directly regulates the transcriptional activities of the *COL8A2* and *ZP4* genes in CECs.

ZP4-expressing CECs highly expressed TFAP2B and COL8A2

Because ZP4 has been reported as one of the cell-surface proteins (24), we isolated ZP4-expressing CECs using an anti-ZP4 antibody by FACS. To confirm the relationship

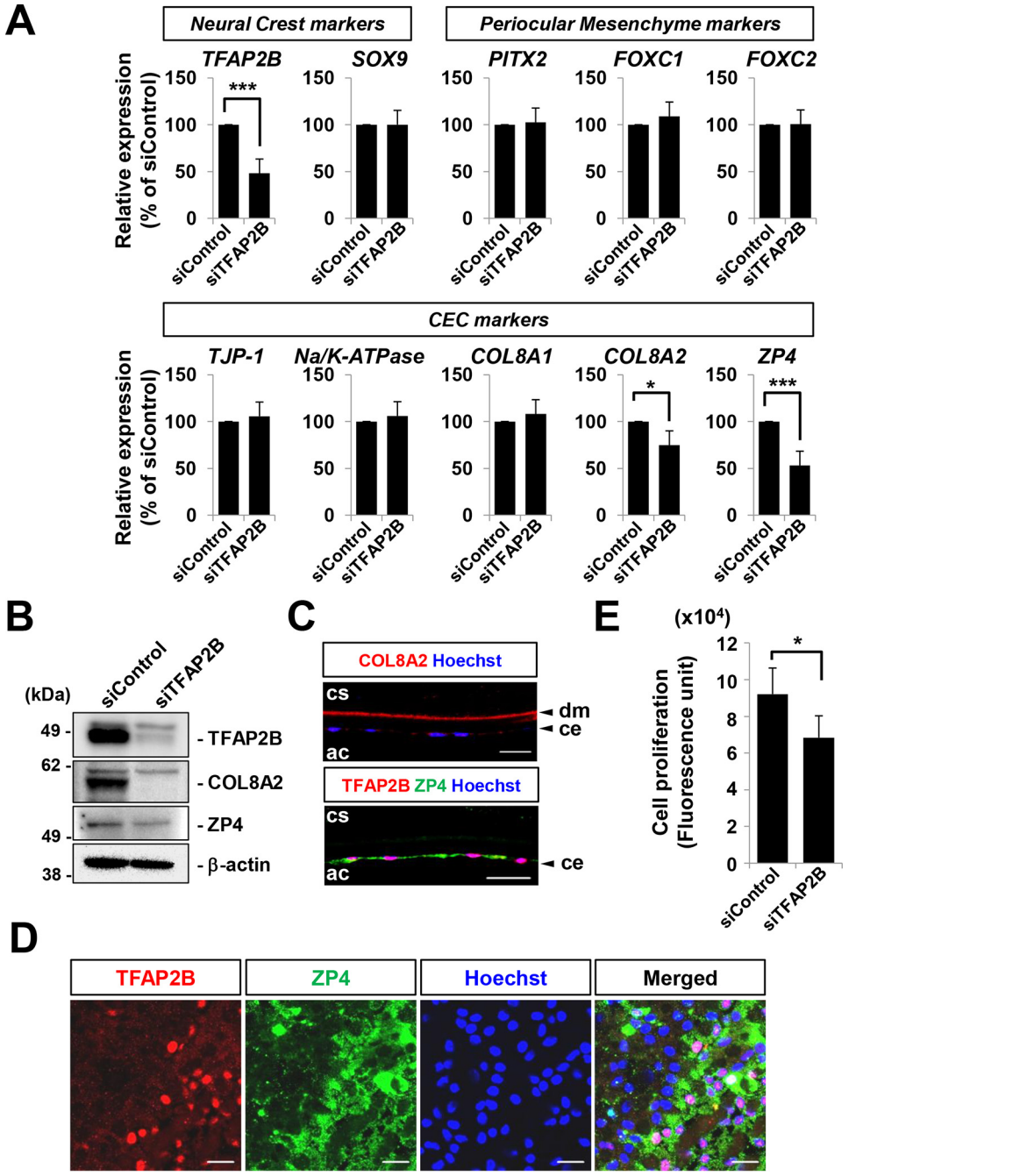

Figure 3. Repression of TFAP2B in human cultured CECs. The siRNA-mediated knockdown in human cultured CECs was performed with control siRNA (*siControl*) and TFAP2B siRNA (*siTFAP2B*) for 48 h. *A*, real-time qRT-PCR of the neural crest marker SOX9; periocular mesenchyme markers PITX2, FOXC1, and FOXC2; and CEC markers TJP1 (ZO-1), Na^+/K^+-ATPase, COL8A1, COL8A2, and ZP4 following TFAP2B siRNA treatment. The expression levels were normalized to those of the siControl-treated CECs ($n = 4$). *B*, Western blotting of TFAP2B siRNA–treated cultured CECs. *C*, immunofluorescence images of COL8A2 (*left panel*, *red*) and ZP4 (*right panel*, *green*) in human corneal sections. *D*, whole-mount immunofluorescence images of TFAP2B (*red*) and ZP4 (*green*). The TFAP2B-positive cells strongly expressed ZP4 protein in the human corneal endothelium. Hoechst 33342 (*blue*) was used to stain the nucleus. *E*, cell proliferation assay using AlamarBlue reagent of siControl- and siTFAP2B-treated CECs ($n = 6$, duplicate three donors). The cells were seeded at 3,000 cells/well in a 96-well plate and analyzed after 2 days. The data are shown as the mean ± S.D. (*error bars*) *, $p < 0.05$; ***, $p < 0.001$. *Scale bars*, 20 μm. *cs*, corneal stroma; *ac*, anterior chamber; *dm*, Descemet's membrane; *ce*, corneal endothelium.

between the expression of TFAP2B and ZP4, we performed purification experiments with cultured human CECs using an anti-ZP4 antibody. Among the cultured CECs, 14.4% of the cells were positively stained by ZP4 (Fig. 5*A*). qRT-PCR analysis demonstrated that the ZP4-expressing cells had significantly higher expression levels of TFAP2B and COL8A2, but those of COL8A1 were not significantly different (Fig. 5*B*). When ZP4-positive and ZP4-negative cells were respectively cultured, it was found that TFAP2B expression was high in ZP4-positive cells by immunofluorescence (Fig. 5*C*). Furthermore, ZP4-positive cells had a higher proliferative ability than ZP4-negative cells (Fig. 5, *D* and *E*). These data suggest that ZP4-expressing CECs may have a high proliferative potential.

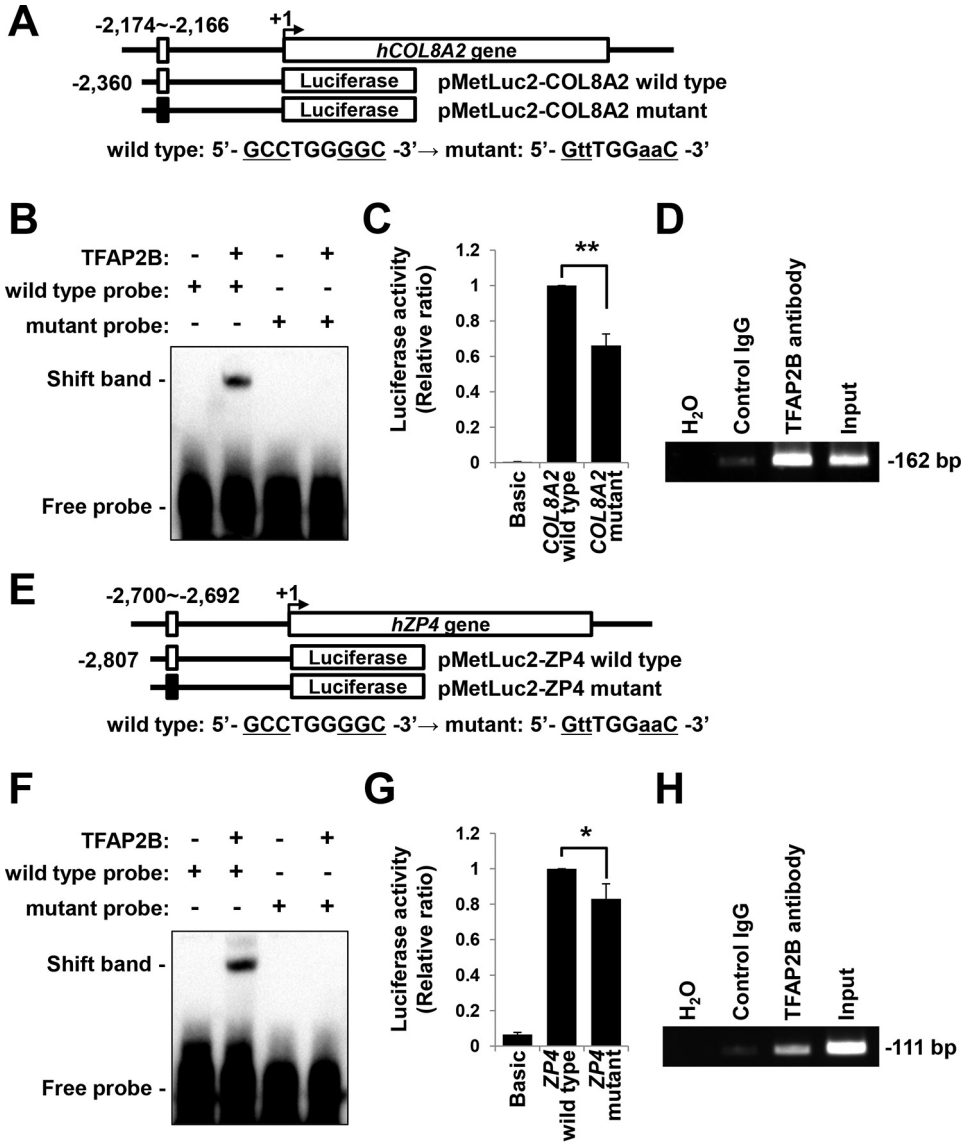

Figure 4. Transcriptional activities of the *COL8A2* and *ZP4* promoters with TFAP2B. *A* and *E*, scheme of the luciferase reporter vectors of the human *COL8A2* and *ZP4* promoters. Mutations in the TFAP2B-binding site are shown in *lowercase letters*. *B* and *F*, EMSA of candidate TFAP2B-binding site of *COL8A2* and *ZP4* promoters. The shifted bands of the DNA–TFAP2B protein complexes were only observed in WT sequences of *COL8A2* and *ZP4*. *C* and *G*, luciferase assays. TFAP2B-overexpressing 293T cells were transfected with the luciferase reporter vector containing the promoter regions of the *COL8A2* and *ZP4* genes. The luciferase activities were compared between the WT (*COL8A2* or *ZP4*) and TFAP2B-binding sequence mutant (*COL8A2* mutant and *ZP4* mutant) luciferase vectors. The data were normalized to the luciferase activity of the WT. *D* and *H*, ChIP. DNA–protein complexes of cultured CECs were immunoprecipitated with IgG control or anti-TFAP2B antibody. Purified DNA was amplified using PCR and separated on an agarose gel. The input samples were used as a control. The data are shown as the mean ± S.D. (*error bars*) ($n = 4$). *, $p < 0.05$; **, $p < 0.01$. *Scale bars*, 20 μm.

Discussion

We previously identified TFAP2B as one of the corneal endothelium–specific transcription factors (13). However, it was not clear which genes were transcriptionally controlled by TFAP2B in CECs. Here, we demonstrate that the expression levels of functional markers such as ZO-1 and Na^+/K^+-ATPase and periocular mesenchymal markers such as PITX2 and FOXC1 were not affected by the knockdown of TFAP2B in CECs. It is possible that TFAP2B may play a different role between corneal endothelium–specific genes such as *COL8A2* and *ZP4* and functional markers that are not specific to CECs such as ZO-1 and Na^+/K^+-ATPase. However, it was previously reported that TFAP2B is required for the expression of ZO-1 in the corneal endothelium and is responsible for the barrier function during corneal development (25).

Systemically, the expression of TFAP2B has been found in various tissues such as the skin, spinal cord, renal tubular epithelia, facial mesenchyme, and cornea (26). Mutation of the *TFAP2B* gene is known to cause Char syndrome, an autosomal dominant disease characterized by facial dysmorphism (27). Additionally, *TFAP2B*-deficient mice die in the postnatal period due to polycystic kidney disease (28). Recently, in the eye, the *TFAP2B* gene was suggested to be an early-onset glaucoma-causing gene in an analysis of periocular mesenchyme–specific *TFAP2B* knockout mice (29). However, disorders associated with *TFAP2B* in CECs have not been reported.

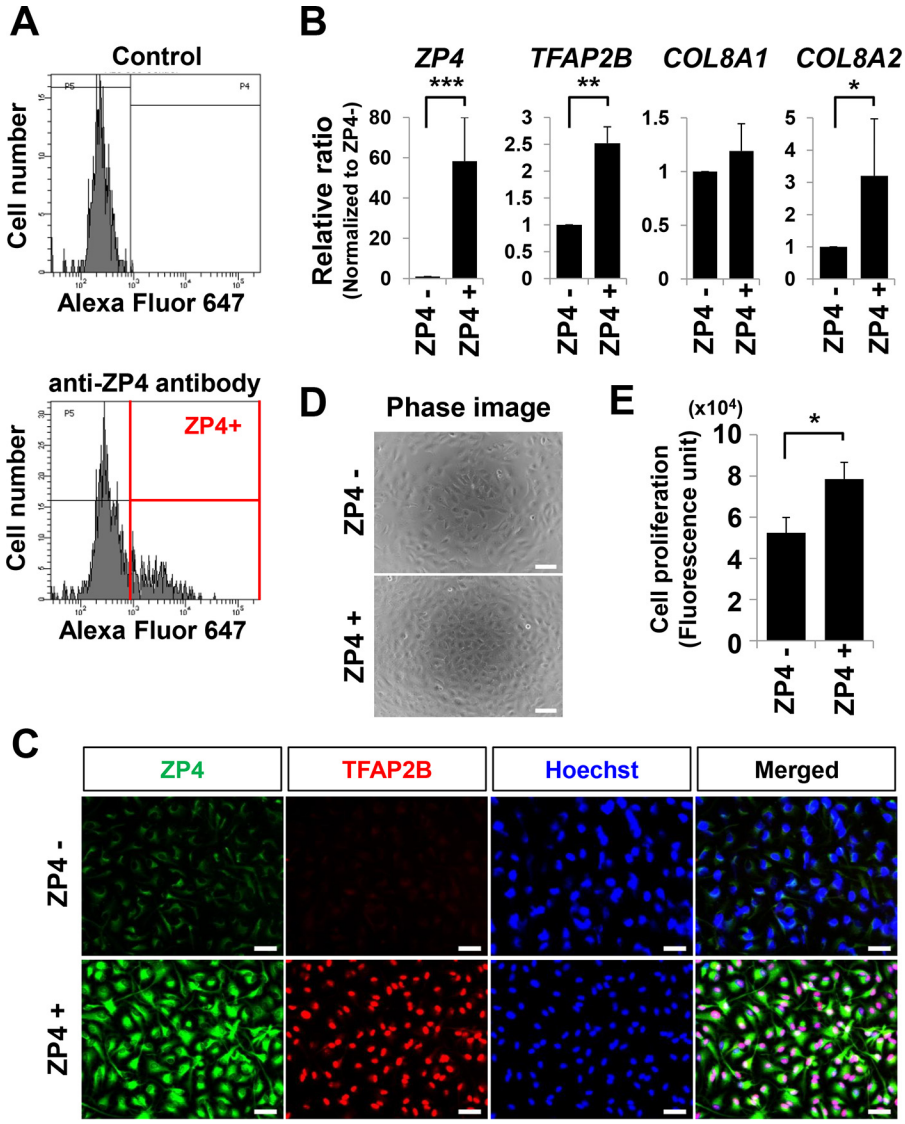

Figure 5. Isolation of ZP4-expressing cells in CECs. *A*, cultured human CECs were separated into ZP4-negative or ZP4-positive (*ZP4*+) populations by FACS. *B*, expression of corneal endothelium–related genes in the sorted cells. ZP4-positive cells (*ZP4* +) highly expressed ZP4, TFAP2B, and COL8A2 compared with ZP4-negative cells (*ZP4*−). The expression levels were normalized to those of ZP4-negative cells. The cells collected by FACS were seeded at 20,000/per well in a 96-well plate and analyzed after 2 or 7 days. *C*, immunostaining of cells cultured for 7 days after cell sorting. Isolated ZP4-positive or ZP4-negative cells were cultured (*D*), and cell proliferation assays were performed after 2 days (*E*). Data are shown as the mean ± S.D. (*error bars*) ($n = 4$). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *Scale bars*, 100 μm.

COL8A2 is one of the major components of the Descemet's membrane along with COL8A1 and is generated from CECs (30), and mutations of the *COL8A2* gene were reported to cause Fuchs endothelial corneal dystrophy (31, 32). Moreover, the *COL8A1*/*COL8A2* double-null mouse phenotype was found to be associated with anterior segment abnormality in the cornea (33). We speculate that the relationship between TFAP2B and COL8A2 may be crucial in the developmental stage in CECs.

We also identified *ZP4* as a target gene of TFAP2B wherein TFAP2B binds to the *ZP4* promoter region and directly induces the expression of the ZP4 protein. Furthermore, we found that ZP4 is a specific surface marker of human CECs (20). Several corneal endothelial markers have previously been isolated such as sodium bicarbonate transporter–like protein 11, COL8A2, cysteine- and tyrosine-rich protein 1, glypican-4, cluster of differentiation 200 (CD200), and ZP4 (34, 35). We previously showed that ZP4 was expressed in human CECs, oocytes, and other eye tissues but had little to no expression elsewhere in the body (20). Although the specific function of ZP4 in CECs remains unclear, our data indicate that it is expressed in CECs with high proliferative capacity. We were able to isolate ZP4-expressing cells via FACS and found that ZP4 protein–expressing cells correlated with the increased expression of TFAP2B and COL8A2.

Known corneal endothelial stem/progenitor markers such as p75, Ki67, leucine-rich repeat– containing G protein– coupled receptor 5, nestin, and telomerase are all localized in the peripheral region of the corneal endothelium (9, 36–38). Of these, we previously reported that p75 was expressed in human corneal endothelial progenitor cells as a neural crest stem cell marker (9). p75-expressing cells *in vivo* are diffusely distributed

in the center region and are more concentrated in the peripheral region. In the present study, *in vivo*, we found that TFAP2B was distributed within the entire corneal endothelium with especially strong expression in the peripheral region. Subsequently, *in vitro*, we also showed that CECs expressing TFAP2B have high cell proliferation ability and that ZP4-expressing cells express TFAP2B protein and have high proliferative ability as well. In a previous report, the AP-2 family was shown to be suppressed through cell proliferation via p21WAF1/CIP1; however, TFAP2B may promote cell proliferation in CECs and some cancer cells such as nonsmall cell lung cancer cells and breast cancer cells (39–41). The cells with high expression of TFAP2B tended to have a smaller cell nucleus size in *in vivo* immunostaining (Fig. 1*B*) and ZP4 sorting experiments (Fig. 5*C*). The nucleus in the peripheral regions is smaller than that in the central regions, and corneal endothelial stem/progenitor cells are known to exist in the peripheral region (36–38). These data suggested that TFAP2B is one of the transcription factors necessary for not only determining differentiation into CECs but also maintaining high proliferative capacity.

We demonstrated that the expression levels of functional markers such as ZO-1 and Na^+/K^+-ATPase were not affected by the knockdown of TFAP2B in CECs. Transcriptional regulation of the pump and barrier functional genes in the corneal endothelium has not been sufficiently clarified. If detailed analysis of transcription factors of functional markers is carried out, the mechanism of corneal endothelial development and homeostasis maintenance may become clear.

In conclusion, we demonstrated that TFAP2B is expressed in the corneal endothelium and transcriptionally regulates corneal endothelium–related genes. In the future, research on corneal endothelium transcription factors may provide information on the corneal endothelial differentiation mechanism and homeostatic maintenance. These factors may also be important in regenerative medicine using cell-based therapy with corneal endothelial cells and pluripotent stem cells.

Experimental procedures

Research-grade human corneal tissues

Research-grade sclerocorneal tissues from cadaver donors considered unsuitable for clinical use were obtained from the eye bank CorneaGen Inc. (Seattle, WA). The use of sclerocorneal tissues strictly followed the tenets of the Declaration of Helsinki. Approval of the experimental protocols was exempted by the Osaka University Review Board.

Animal study

The usage of mice was approved by the animal ethics committee of Osaka University. Mice were anesthetized with sodium pentobarbital (200 mg/kg) after which perfusion fixation was performed. The mouse tissues were fixed in 4% paraformaldehyde in PBS at 4 °C overnight. Frozen sections were prepared from tissues embedded in OCT Compound (Sakura Finetek).

Antibodies

The polyclonal anti-human ZP4 antibody was produced in rabbits using immunization with a synthetic peptide (N-CPMDLLARDAPDTDW-C) against a portion (NP_067009, amino acids 128–142) of the human ZP4 protein. For the production of the mouse monoclonal anti-human ZP4 antibody, mice were injected five times with the synthetic peptides. Hybridomas producing anti-human ZP4 antibody were selected by screening using ELISA and Western blotting. The mAb was purified with a PD-10 column (GE Healthcare) and HiTrap SP HP (GE Healthcare). Anti-TFAP2B antibody was purchased from Cell Signaling Technology (catalog number 2509). Anti-COL8A2 antibody was purchased from Abgent (AP11859b). Anti-ZO-1 antibody (catalog number 33-9100) was purchased from Thermo Fisher Inc. Anti-β-actin (A5441) antibody was purchased from Sigma-Aldrich.

Culture of human CECs

The human CECs containing Descemet's membranes were carefully peeled with tweezers under a microscope and were treated with Stem Pro Accutase (Thermo Fisher Scientific Inc.) at 37 °C for 30 min. The dissociated CECs were cultured using a previously reported modified culture method with serum-free medium (42). The cells were seeded at ~3,000 cells/well in laminin-511E8 (iMatrix-511, Nippi, Inc.)– coated (0.1 $\mu g/cm^2$) culture plates with Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Thermo Fisher Scientific Inc.) supplemented with 10% KnockOut Serum Replacement (Thermo Fisher Scientific Inc.), 1× insulin-transferrin-selenium (Thermo Fisher Scientific Inc.), 4 ng/ml basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd., Osaka, Japan), leukemia inhibitory factor (1:1,000; Wako Pure Chemical Industries), 20 μg/ml streptomycin, and 20 units/ml penicillin (Thermo Fisher Scientific Inc.). For each of the subsequent experiments described below, we used the cultured cells up to three times to ensure that they retained their endothelial properties (*e.g.* pump function).

Semiquantitative RT-PCR and quantitative PCR

The corneal endothelial tissues containing peripheral and central areas were carefully collected from the sclerocorneal tissue. Total RNA was isolated using a RNeasy Plus Micro kit (Qiagen), and cDNA was then synthesized using SuperScript III for qRT-PCR (Thermo Fisher Scientific Inc.). Neural retinal cDNA was used as positive control for RT-PCR. The cDNAs were amplified by PCR and electrophoresed on an agarose gel. The primer sequences and their product lengths are presented in Table S1. Real-time quantitative PCR was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.) using TaqMan probes (Thermo Fisher Scientific Inc.). The catalogue numbers of the TaqMan probes used are shown in Table S2.

Immunofluorescence

The human corneal tissues and cultured CECs were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. The specimens were treated with 5% normal donkey serum in Tris-buffered saline (TBS) containing 0.3% Triton X-100 (Sigma-Aldrich) at room temperature for 60 min and incubated with the primary antibodies listed above. Fluorescence images were observed and photographed under a fluorescence micro-

scope (AxioObserver Z1, Carl Zeiss Microscopy GmbH, Jena, Germany) or a confocal microscope (LSM710, Carl Zeiss Microscopy GmbH). The relative ratio of the fluorescence intensity between the fluorescence of TFAP2B and fluorescence of Hoechst 33342 was calculated using ZEN2012 software (Carl Zeiss Microscopy GmbH). The peripheral region and central region of the human corneal endothelium were defined as reported by He *et al.* (37).

siRNA transfection

Cultured human CECs were transfected with 10 nM siRNA (Silencer Select siRNA, Thermo Fisher Scientific Inc.) using Lipofectamine RNAiMAX (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The CECs were seeded at 10,000 cells/cm^2. The sequences of the siRNA against TFAP2B were 5′-AGUUCAACUUCGAAGUACAtt-3′ in the sense direction and 5′-UGUACUUCGAAGUUGAACUga-3′ in the antisense direction. Silencer Select control siRNA 1 (Thermo Fisher Scientific Inc.) was used as a nonsilencing control for siRNA. Forty-eight hours after the siRNA transfection, the CECs were used for subsequent experiments.

Western blotting

The cells were directly lysed with a sample buffer (2× NuPAGE LDS Sample Buffer, Thermo Fisher Scientific Inc.) with 2.5% β-mercaptoethanol and sonicated. The lysates were denatured at 95 °C for 5 min, then electrophoresed on a 4–12% gradient polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. The membranes were then blocked with 5% nonfat milk diluted in TBS containing 0.05% Tween 20 (TBS-T) at room temperature for 60 min and blotted with primary antibodies at 4 °C overnight. After being washed three times with TBS-T, the membranes were again blotted with horseradish peroxidase–conjugated secondary antibody diluted in 2.5% nonfat milk–containing TBS-T. After washing three times with TBS-T, the chemiluminescence signal of the membrane was detected using ECL Prime Western Blotting Detection Reagent (GE Healthcare Ltd.), photographed using a ChemiDoc XRS (Bio-Rad), and analyzed using commercial software (Quantity One analysis software, Bio-Rad).

EMSA

EMSA was performed according to the manufacturer's protocols (LightShift Chemiluminescent EMSA kit, Thermo Fisher Scientific Inc.). Briefly, 5′-biotin–labeled double-strand oligonucleotides (Table S1) were incubated with recombinant TFAP2B protein (H00007021, Abnova Corp.) at 25 °C for 30 min. Then the samples were loaded onto a 6% polyacrylamide gel and transferred onto nylon membranes. The membranes and DNA were cross-linked at 150 mJ/cm^2 using a commercial UV-light cross-linking instrument (GS Gene Linker, Bio-Rad) and incubated with horseradish peroxidase–conjugated streptavidin. The light signals were detected using a ChemiDoc XRS.

Luciferase assay

The upstream regions of the human *COL8A2* and *ZP4* genes were amplified by PCR and inserted into the EcoRI site of the empty luciferase vector (pMetLuc2-basic vector, Takara Bio Inc., Shiga, Japan) using a commercial cloning kit (In-Fusion HD Cloning kit, Takara Bio Inc.). Site-directed mutagenesis at the AP-2–binding motif of the human *COL8A2* and *ZP4* genes was performed using a commercial kit (PrimeSTAR Mutagenesis Basal kit, Takara Bio Inc). To clone TFAP2B, cDNA from human CECs was amplified by PCR using primers for TFAP2B cDNA and then subcloned into an entry vector (pENTR/D-TOPO, Thermo Fisher Scientific Inc.). Then TFAP2B cDNA in pENTR-TFAP2B was transferred to the pLenti7.3/V5-DEST-Gateway vector using the *in vitro* recombination enzyme Gateway LR Clonase II Enzyme Mix reagent (Thermo Fisher Scientific Inc.) to obtain the TFAP2B-expressing vector (pLenti7.3-TFAP2B). 293T cells (1×10^4 cells) were transfected with 100 ng of the above reporter vector, 100 ng of the pSEAP control vector (Takara Bio Inc.), and 100 ng of the pLenti7.3-TFAP2B vector using Lipofectamine 3000 reagent (Thermo Fisher Scientific Inc.). Twenty-four hours after the transfection, the activities of both the secreted luciferase and secreted alkaline phosphatase in the cultured medium were measured with a commercial luciferase assay reagent kit (Ready-To-Glow Dual Secreted Reporter Assay, Takara Bio Inc.) using a plate reader (ARVO X4, PerkinElmer Life Sciences). The luciferase activity data were normalized with those of SEAP activity. The primer sequences are shown in Table S1.

ChIP assay

The ChIP assay was performed according to the manufacturer's protocols (EZ-ChIP, Merck KGaA). Briefly, cultured CECs were fixed in 1% formaldehyde at 37 °C for 10 min, then quenched with a final concentration of 125 mM glycine at room temperature for 5 min, and fragmented using a sonicator (Covaris Inc.). The samples were immunoprecipitated with anti-TFAP2B (1:100) antibody and normal rabbit IgG. The coimmunoprecipitated DNA was purified and amplified by PCR using the primer pairs against the upstream regions of the human *COL8A2* and *ZP4* promoters. The primer sequences are shown in Table S1.

FACS

Cultured CECs were enzymatically detached using Stem Pro Accutase at 37 °C for 10 min. The dissociated cells were stained with serum containing anti-ZP4 antibody diluted in PBS with 2% fetal bovine serum (FBS) (at 1:1,000) at 4 °C for 30 min. After being washed in PBS containing 2% FBS, the cells were again stained with an Alexa Fluor 647–conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific Inc.) diluted in PBS containing 2% FBS (at 1:2,000) at 4 °C for 30 min. Preimmunized rabbit serum was used as a negative control. Analysis and cell sorting were performed using a BD FACS Aria II (BD Biosciences).

Cell proliferation assay

The cells were treated with a medium containing Alamar-Blue reagent (AbD Serotec) according to the manufacturer's protocol. After 4 h, the culture supernatants were collected and analyzed with a plate reader.

Statistical analysis

Means ± S.D. were calculated using the *t* test or Tukey–Kramer test for multiple comparisons. All statistics were calculated using commercial statistical software (JMP 11.2.0, SAS Institute Inc., Cary, NC). A *p* value less than 0.05 was considered statistically significant.

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