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Spatiotemporal gene targeting in the mouse corneal endothelium

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Abstract:

PURPOSE: The inducible Cre-ERT2 recombinase system allows for temporal control of gene targeting, and it is useful to studying adult function of genes that have critical developmental roles. The Zeb1$^{flox/flox}$: UBC-CreERT2 mouse was generated to conditionally target Zeb1 to investigate its role in mesenchymal transition in the mouse corneal endothelium *in vivo*.

MATERIALS AND METHODS: Hemizygous UBC-CreERT2 mice were crossed with homozygous mice harboring loxP-flanked Zeb1 alleles (Zeb1$^{flox/flox}$) to generate the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse. 4-hydroxytamoxifen (4-OHT) exposure leads to excision of exon 6 of Zeb1, resulting in a loss function allele in the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse. Intracameral 4-OHT injection further isolates Zeb1 targeting to the anterior chamber. Mesenchymal transition and induction of Zeb1 expression in the corneal endothelium was achieved using FGF2 in *ex vivo* organ culture. Gene expression was analyzed by semi-quantitative reverse transcription-polymerase chain reaction and by immunoblotting in the mouse corneal endothelium *in vivo*.

RESULTS: Following Cre-mediated targeting of Zeb1 by intracameral 4-OHT injection in Zeb1$^{flox/flox}$: UBC-CreERT2 mice, FGF2 treatment in *ex vivo* organ culture resulted in abrogation of Zeb1 mRNA and protein expression in the corneal endothelium.

CONCLUSION: The data show Zeb1, a critical mediator of fibrosis in corneal endothelial mesenchymal transition, can be targeted by intracameral injection of 4-OHT in the mouse corneal endothelium *in vivo*. These results suggest that genes with critical developmental roles can be targeted at a specific time in the corneal endothelium to study its role in adult disease using an inducible Cre-Lox strategy.

Keywords:

Conditional targeting, corneal endothelium, mouse

Introduction

Conditional gene targeting is a powerful technique that allows for studying gene function in one tissue without affecting expression in other tissues.[1] This is particularly useful for studying adult function of a gene if knocking it out of the whole organism leads to an embryonic or perinatal lethal phenotype. Tissue-specific activation of Cre recombinase (Cre) paired with flanking a gene of interest with *loxP* sequences can be used to avoid the above-mentioned limitations observed in some constitutive knockout models.[2] Furthermore, temporal control of Cre activation can prevent potential developmental impact of constitutively knocking out a gene.[3-5] The Cre recognizes two *loxP* sequences and mediates excision of the intervening DNA sequence and allows for gene targeting in specific tissue and specific time using an inducer such as 4-hydroxytamoxifen (4-OHT).[6-8] The second generation of tamoxifen inducible- Cre is composed of Cre fused with a mutant human estrogen receptor harboring a G400V/M543A/L544A triple mutation in the ligand binding domain (ERT2) which is more sensitive and specific for 4-OHT than the wildtype estrogen receptor.[3,9,10]

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The fusion protein is normally located in the cytoplasm bound to heat shock protein 90 (HSP90). Binding of 4-OHT to CreERT2 leads to its release from HSP90, translocation into the nucleus, and excision of the target sequence between the *loxP* sites.[3,6,11]

Cell proliferation, migration, and fibrosis, critical for restoring tissue integrity in wound healing, are features observed in mesenchymal transition. Mesenchymal transition is a process where cells lose their polarity, assume a fibroblastic phenotype, and exhibit enhanced cell proliferation, migration, and type I collagen secretion, and corneal endothelial cells (CECs) can undergo mesenchymal transition (EnMT) in response to severe injury or inflammation.[12-15] Unlike other tissues where restoration of tissue integrity is beneficial, EnMT-induced fibrosis leads to retrocorneal membrane (RCM) formation with subsequent vision loss in the cornea and is undesirable. Zinc finger E-box binding homeobox 1 (Zeb1) plays a critical role in induction of fibrosis in EnMT in human and mouse corneal endothelium *ex vivo*.[16] Surgical injury can induce FGF2 expression and EnMT in the mouse corneal endothelium *in vivo*, and siRNA-mediated knockdown of Zeb1 can inhibit injury-dependent RCM formation in the mouse corneal endothelium *in vivo*.[17,18]

Although targeting Zeb1 by siRNA or blocking its interaction YAP1 with verteporfin or CtBP by NSC95397 and MTOB is possible, these approaches are fraught by non-specific or off target effects.[19,20] A genetic approach to targeting Zeb1 is more desirable, but the Zeb1 null mouse cannot be used to study Zeb1 function in EnMT in the adult corneal endothelium because knocking out Zeb1 leads to a perinatal lethal phenotype due to numerous developmental abnormalities.[21] These limitations led us to generate the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse to allow for temporal and spatial control of Zeb1 targeting. In the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse, CreERT2 is expressed under the control of Ubiquitin C promoter, and intracameral injection of 4-OHT allows for temporal targeting of Zeb1 in the mouse corneal endothelium *in vivo*. Here, we report that the Cre-*loxP* approach can be used to achieve conditional gene targeting in the mouse corneal endothelium *in vivo*.

Materials and Methods

Animal husbandry

All mouse experiments were performed in accordance with the protocol approved by University of Southern California Institutional Animal Care and Use Committee (IACUC protocol number 21213). The mice were housed in clear, air-filtered cages with 12-h light/dark cycle and ad lib feeding. C57BL/6 and C57BL/6 UBC-CreERT2 mouse breeding pairs were purchased from Jackson Laboratories (Sacramento, CA, USA) and C57BL/6 Zeb1$^{flox/flox}$ was acquired from Prof. Marc Stemmler.[22] Zeb1$^{flox/flox}$: UBC-CreERT2 mice were generated by crossing Zeb1$^{flox/flox}$ mice with UBC-CreERT2 mice. Colonies used in this study were bred in-house. Mice between ages of 12 and 14 weeks were used for all experiments. Mice were anesthetized by intraperitoneal injection of ketamine (60–70 mg/kg) and xylazine (5–10 mg/kg), and they were euthanized by cervical dislocation.

Materials

FGF2 was purchased from Cell Signaling Technology (Danvers, MA, USA). 4-hydroxytamoxifen was obtained from Tocris Bioscience (Minneapolis, MN, USA). Anti-β-actin (42 kDa, A5316) and peroxidase-conjugated secondary antibodies were obtained from MilliporeSigma (Burlington, MA, USA). Anti-ZEB1 (124 kDa, PA5–40350) antibody was purchased from Thermo Fisher (Waltham, MA, USA).

Genotyping

Genomic DNA extraction and amplification with gene-specific primers were performed using Phire Tissue Direct polymerase chain reaction (PCR) master mix (ThermoFisher Scientific). Primers for the detection of Cre-ERT2 (475bp): 5′-GACCTGACCCGTTCTGTTG-3′ (forward) and 5′-AGGCAAATTTTGGTGTACGG-3′ (reverse). Primers for the detection of Zeb1$^{+/+}$, Zeb1$^{flox/flox}$ and Zeb1$^{del/del}$ alleles: Zeb1 F1, 5′-CGTGATGGAGCCAGAATCTGACCCC-3′; Zeb1 R1, 5′-GCCCTGTCTTTCTCAGCAGTGTGG-3′; Zeb1 F2, 5′-GTCACTTCTACACTGGCAGCTA-3′; Zeb1 R2, 5′-GCCATCTCACCAGCCCTTACTGTGC-3′. PCR conditions for genotyping were as follows: 2 min at 94°C, followed first by 20 s at 94°C, 15 s at 65°C, 10 s at 68°C for 10 cycles, then by 15 s at 94°C, 60 s at 15°C, 10 s at 72°C for 28 cycles, and with a final extension for 4 min at 72°C. DNA fragments were separated by electrophoresis on a 1.5% agarose gel to access whether the deletion of Zeb1 exon 6 and the presence of CreERT2 and Zeb1$^{flox/flox}$. The animals used in this study were UBC-CreERT2 hemizygotes and Zeb1$^{flox/flox}$ homozygotes.

4-hydroxytamoxifen preparation and treatment

4-OHT was dissolved in 100% ethanol (molecular biology grade) at 20 mg/ml and diluted with corn oil (MilliporeSigma) for final concentration 10 uM. For intracameral injections, 3 µl of aqueous humor was aspirated and 3 µl of 10 µM 4-HOT was injected into the anterior chamber at the limbus using a 30-gauge needle daily for three consecutive days. The vehicle-injected contralateral eye was used as control. Mice were euthanized 7 days′ post first intracameral injection of 4-OHT, and their eyes were enucleated. All procedures were performed under direct visualization

using an operating microscope, and care was taken not to injure the lens. Corneas were excised from the enucleated eyes, the endothelium was stripped from the excised corneas, and the stripped endothelium was then processed for reverse transcription (RT)-PCR and immunoblotting.

Semi-quantitative reverse transcription-polymerase chain reaction analysis

The corneas were isolated from eyes of 6 mice per experimental group and the corneal endothelium-Descemet membrane complex was stripped using jeweler's forceps under a dissecting microscope. Total RNA was extracted from mouse corneal endothelium and RT-PCR was performed as previously described.[18] Briefly, cDNA was synthesized with 0.5 μg of RNA by utilizing iScript reverse transcriptase (Bio-Rad, Hercules, CA, USA) and oligo (dT) primer. Reverse transcription was performed at 42°C for 90 min. Then, the first strand cDNA equivalent to 0.05 μg of starting RNA from each sample was amplified using the specific primer pairs. The specific primers and PCR conditions used are shown in Table 1. Standard PCR conditions were as follows: 5 min at 94°C, followed by 30 s at 94°C, 30 s at 53°C, 30 s at 72°C, and a final extension for 4 min at 72°C. PCR cycles were optimized to ensure that the product amplification fell within the linear phase of amplification and annealing temperature were adjusted depending on the PCR primers [Table 1]. RT-PCR amplification of β-actin transcript was used as loading control. The amplified products were separated on a 1.5% agarose gel electrophoresis, visualized by Gel-Red staining, and the band intensity was analyzed using Image Lab program from Bio-Rad. All target PCR products were verified by DNA sequencing.

Protein preparation, protein assay, SDS PAGE, and immunoblotting analysis

All assays were performed following previously reported protocols.[16,23] The following gel concentrations were used to separate proteins: 10% polyacrylamide gel for β-actin and 8% polyacrylamide gel for ZEB1. The corneas were isolated from eyes of 18 mice per experimental group and the corneal endothelium-Descemet membrane complex was stripped using jeweler's forceps under a dissecting microscope. For protein purification from *ex vivo* corneal endothelium, cells in the tissue were lysed with RIPA lysis buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) *in situ*. Total protein was purified and concentrated with Amicon ultra centrifugal filter devices (MilliporeSigma), according to the manufacturer's instructions. Briefly, cell lysates were applied to the Amicon ultra 10 K centrifugal device (molecular weight cutoff 10 K), and then spin down at 14,000 × g for 30 min. To recover the concentrated protein, the Amicon ultra filter device placed upside down in a clean tube which was then centrifuged again for 2 min at 1000 × g to transfer the concentrated protein from the device to the clean tube. Purified total proteins were used to for analysis of immunoblotting.

Results

Generation of Zeb1$^{flox/flox}$: UBC-CreERT2 mice

Mice harboring 2 alleles the *Zeb1* gene with exon 6 flanked by *loxP* sequences (Zeb1$^{flox/flox}$) were crossed to hemizygous UBC-CreERT2 transgenic mice to generate Zeb1$^{flox/flox}$: UBC-CreERT2 mice [Figure 1a]. To induce nuclear translocation of the CreERT2 fusion protein and excision of exon 6,[3,6,11] 4-OHT was injected in the anterior chamber of Zeb1$^{flox/flox}$: UBC-CreERT2 and Zeb1$^{flox/flox}$ mice. Seven days after intracameral injections of 4-OHT or vehicle, genotyping was performed on purified genomic DNA isolated from corneal endothelium of Zeb1$^{flox/flox}$: UBC-CreERT2 and Zeb1$^{flox/flox}$ mice. In the corneal endothelium of mice that received vehicle injections, PCR products of 295 bp with the F1-R1 primer pair and 512 bp with the F2-R2 primer pair were detected, while the 367 bp product with the F1-R2 primer pair was not detected [Figure 1b]. The corneal endothelium from Zeb1$^{flox/flox}$: UBC-CreERT2 mice injected with 4-OHT showed the 367 bp product with F1-R2 primer pair, while no PCR products using F1-R1 and F2-R2 primer pairs were detected [Figure 1b]. Zeb1$^{flox/flox}$ mice injected with 4-OHT showed the same PCR product profile as the vehicle-injected mice.

Table 1: Forward and reverse primer sequences, annealing temperature, and number of cycles performed for reverse transcription-polymerase chain reaction

Gene	Primers	Annealing temperature (°C)	Number of cycles	PCR product size (bp)
β-actin	Forward 5'-GCAGGAGTACGATGAGTCCGG-3'	55	22	296
	Reverse 5'-CTTTGGGGGATGTTTGCTCCA-3'			
Col8a2	Forward 5'-TGAGGGCCTAGTCTCCTTCCC-3'	57	25	357
	Reverse 5'-ACAGCTCCAATCCACAGACGT-3'			
Ktcn	Forward 5'-AACTGAGCTACCTGCGTCTGG-3'	53	25	274
	Reverse 5'-AACTAATACACGTGGCCCCTG-3'			
Zeb1	Forward 5'-CCACAATCGTGGCCATTGCT-3'	57	27	335
	Reverse 5'-TTTGTGTCTCAACAGTGAGC-3'			

Ktcn=Keratocan, *Zeb1*=Zinc finger E-box binding homeobox 1, PCR=Polymerase chain reaction

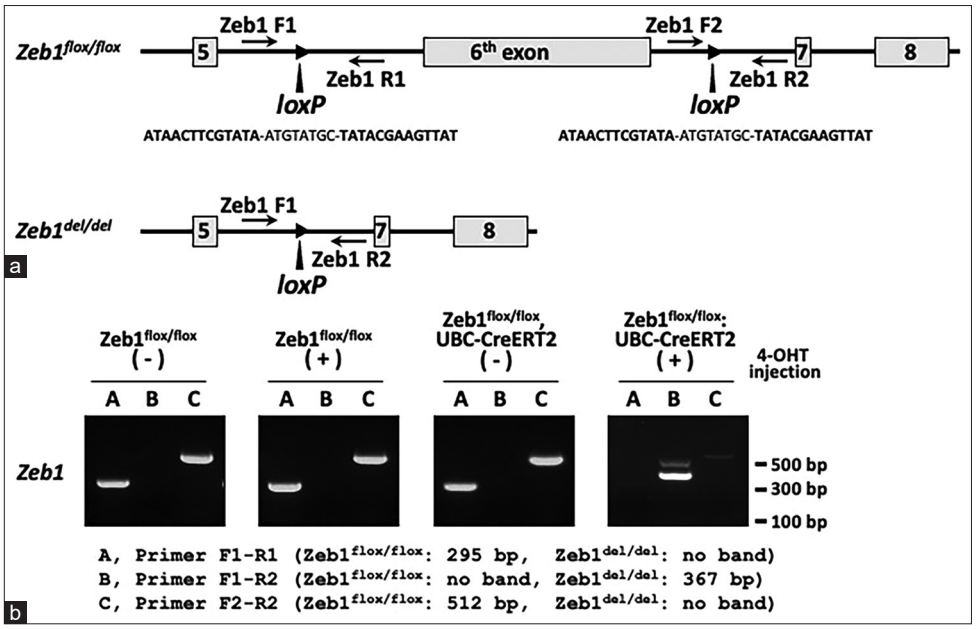

Figure 1: Schematic illustration of the Zeb1 flox allele and deletion of exon 6. (a) Gray boxes indicate exons, and triangles indicate the location of the loxP sites flanking exon 6. The location of F1, F2, R1, and R2 genotyping primers are shown by arrows. Cre recombinase activity leads to excision of exon 6 along with R1 and F2 primer sites. (b) The *loxP* sites were confirmed via genotyping with pair of primers; F1-R1 for proximal site and F2-R2 for distal site. Seven days after intracameral 4-OHT (+) or vehicle (−) injection, the endothelium was isolated and genomic DNA was purified for PCR genotyping. Exon 6 of *Zeb1* was deleted in the corneal endothelium following intracameral 4-OHT injection in Zeb1$^{flox/flox}$: UBC-CreERT2 mice but not in the other mice. In Zeb1$^{flox/flox}$: UBC-CreERT2 mice that received intracameral 4-OHT injection, PCR genotyping with F1-R2 primer pair generated the expected 367 bp product while F1-R1 and F2-R2 reactions did not generate any products due to the loss of R2 and F1 sites following Cre-mediated recombination. In all other groups, F1-R1 and F2-R2 reactions generated the expected 295 bp and 512 bp products respectively while F1-R2 reaction did not generate the expected 367 bp product, indicating *Zeb1* was not altered

Inhibition of FGF2-dependnet Zeb1 expression by intracameral injection of 4-OHT in the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse corneal endothelium

RT-PCR and immunoblotting was performed to determine the effect of exon 6 deletion on *Zeb1* expression. Seven days after intracameral 4-OHT injections, corneas from wildtype, CreERT2 transgenic, Zeb1$^{flox/flox}$, and Zeb1$^{flox/flox}$: UBC-CreERT2 mice were excised and incubated with FGF2 for 7 days in organ culture to induce Zeb1 expression in the endothelium *ex vivo*.[18] Zeb1 RT-PCR using a primer pair, in which the forward primer is located in exon 6 and the reverse primer is located in exon 7, shows that intracameral 4-OHT injection followed by FGF2 treatment in Zeb1$^{flox/flox}$: UBC-CreERT2 mice led to the excision of exon 6 in the *Zeb1* mRNA purified from the corneal endothelium [Figure 2a]. Intracameral 4-OHT injection followed by FGF2 treatment in wildtype, CreERT2 transgenic, and Zeb1$^{flox/flox}$ mice did not lead to excision of exon 6 in the *Zeb1* mRNA purified from the corneal endothelium [Figure 2a]. FGF2 treatment led to an induction of *Zeb1* mRNA expression in all mice, while treatment with vehicle did not induce *Zeb1* mRNA expression. *Col8A2* was used as a marker for CECs, *beta-actin* was used as a loading control, and *Keratocan* was used as a marker for stromal keratocytes[24] [Figure 2a]. Excision of exon 6 leads to a truncated Zeb1 mRNA with a premature stop codon,[22] and this led to an absence of Zeb1 protein in the corneal endothelium of Zeb1$^{flox/flox}$: UBC-CreERT2 mice that received intracameral 4-OHT injection followed by FGF2 treatment [Figure 2b]. Zeb1 protein could be detected in the corneal endothelium of wildtype, CreERT2 transgenic, and Zeb1$^{flox/flox}$ mice that received 4-OHT injection and FGF2 treatment [Figure 2b]. β-actin was used as loading control.

Discussion

The cornea is the anterior transparent tissue of the eye that serves as its main refractive element. Maintenance of transparency is critical for the refractive function, and it is dependent on the coordinated function of its layers, the epithelium, stroma, and endothelium. The corneal endothelium functions as a pump function and regulates corneal hydration, which is critical for maintenance of corneal transparency.[25] Adult human CECs are G_1 arrested but can undergo mesenchymal transition (EnMT) in response to severe injury.[13,26] Endothelial cells that undergo EnMT show increased cell migration, proliferation and secrete collagen type I.[16,23,27] The latter can lead to RCM formation, an opaque, fibrous membrane that can lead to irreversible blindness.[28] Zeb1, a critical mediator of mesenchymal transition in many biological processes, was also identified to be a critical regulator of fibrosis in EnMT.[17] Although siRNA knockdown of Zeb1

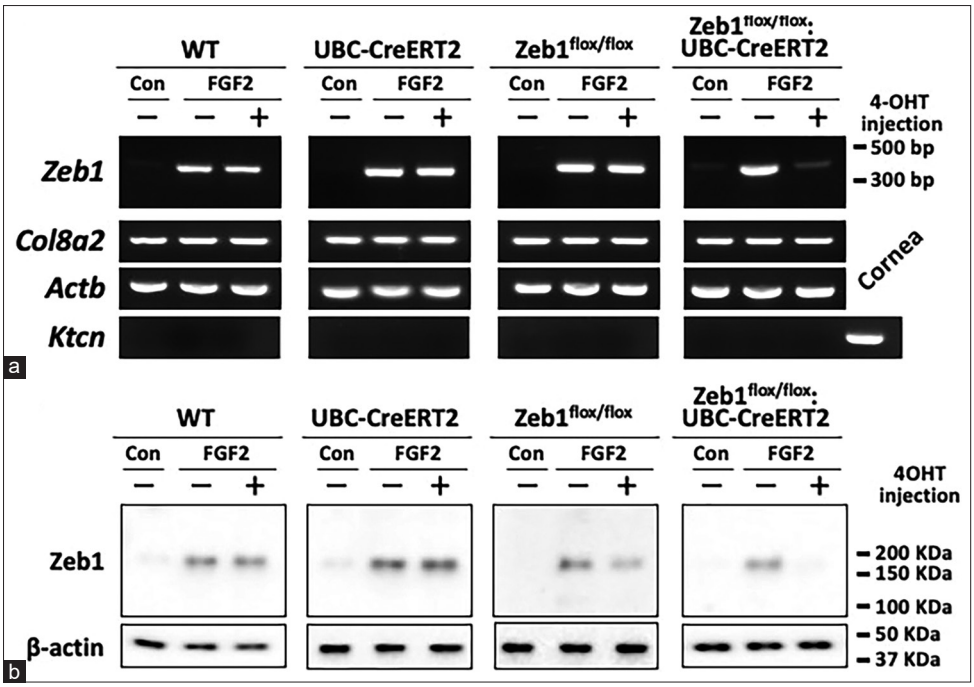

Figure 2: Inhibition of Zeb1 expression by intracameral 4-OHT injection in the corneal endothelium of Zeb1$^{flox/flox}$: UBC-CreERT2 mice. (a) Seven days after intracameral 4-OHT (+) or vehicle (−) injections, the corneal endothelium from wildtype (WT), UBC-CreERT2, Zeb1$^{flox/flox}$, and Zeb1$^{flox/flox}$: UBC-CreERT2 mice, 6 mice per group, were isolated and maintained in organ culture with FGF2 or vehicle (Con) for 7 days *ex vivo*. Total RNA was isolated from the corneal endothelium and RT-PCR was performed for *Zeb1*, *Col8a2*, *ActB*, and *Ktcn*. FGF2 but not vehicle treatment induced *Zeb1* expression in all groups as probed by using the forward primer located in exon 6 and the reverse primer located in exon 7. Intracameral injection of 4-OHT in Zeb1$^{flox/flox}$: UBC-CreERT2 led to loss of exon 6 in the *Zeb1* mRNA in the corneal endothelium. *Col8a2* and *Actb* were used as CEC marker and loading control, respectively. Keratocan (*Ktcn*) was used to control for possible stromal keratocyte and epithelial cell contamination. (b) Seven days after intracameral 4-OHT (+) or vehicle (−) injections, the corneal endothelium from wildtype (WT), UBC-CreERT2, Zeb1$^{flox/flox}$, and Zeb1$^{flox/flox}$: UBC-CreERT2 mice, 18 mice per group, were isolated and maintained in organ culture with FGF2 or vehicle (Con) for 7 days *ex vivo*. Total protein was purified from the corneal endothelium and immunoblotting for Zeb1 and β-actin was performed. FGF2 but not vehicle treatment induced Zeb1 expression in the corneal endothelium of all groups except Zeb1$^{flox/flox}$: UBC-CreERT2 mice that received intracameral 4-OHT injection. Zeb1 protein was not detectable in the corneal endothelium of Zeb1$^{flox/flox}$: UBC-CreERT2 mice that received intracameral 4-OHT injection indicating targeting of Zeb1 in the corneal endothelium following 4-OHT injection

led to decreased RCM formation in the mouse corneal endothelium *in vivo*, a genetic approach to targeting Zeb1 is needed to validate the siRNA knockdown results due to potential siRNA off-target effects. The Zeb1 null mouse develops to term but shows severe defects in neural crest-derived skeletal elements including numerous craniofacial abnormalities, fused ribs, and defects of the sternum, and the embryo does not survive past the postnatal period.[21] The role of Zeb1 in EnMT in the adult mouse cannot be studied using the Zeb1 null mouse because of its perinatal lethal phenotype. This led us to explore using conditional targeting as a genetic approach to studying the role of Zeb1 in EnMT in the adult mouse.

Zeb1$^{flox/flox}$: UBC-CreERT2 mice were generated by crossing Zeb1$^{flox/flox}$ mice to UBC-CreERT2 transgenic mice to take advantage of the Cre-lox system for conditional targeting of Zeb1 in the corneal endothelium. Intracameral injection of 4-OHT in the Zeb1$^{flox/flox}$: UBC-CreERT2 mouse led to excision of exon 6 in the genomic DNA of CECs in the adult mouse [Figure 1b]. Moreover, we did not observe spontaneous Cre activity in the corneal endothelium as evidenced by lack of the 367 bp F1-R2 PCR product in the Zeb1$^{flox/flox}$: UBC-CreERT2 mice that received intracameral injection of vehicle only [Figure 1b]. Excision of exon 6 was not complete since a faint amount of the 512 bp product of F2-R2 reaction can be seen in the corneal endothelium of 4-OHT-injected Zeb1$^{flox/flox}$: UBC-CreERT2 mice [Figure 1b]. We did not observe excision of exon 6 in the Zeb1$^{flox/flox}$ mice. The genomic modification in the corneal endothelium is reflected at the transcriptional level in the 4-OHT-injected Zeb1$^{flox/flox}$: UBC-CreERT2 mice. FGF2 was used in organ culture to stimulate Zeb1 expression. *Zeb1* RT-PCR using mRNA isolated from the corneal endothelium from 4-OHT-injected Zeb1$^{flox/flox}$: UBC-CreERT2 mice showed a severe decrease in the amount intact *Zeb1* mRNA [Figure 2a]. This likely reflects the small amount of intact *Zeb1* gene in the corneal endothelium, and this could be due to an insufficient dosage of 4-OHT in the intracameral injection. Intracameral 4-OHT injection and FGF2 treatment induced *Zeb1* mRNA expression in wildtype, UBC-CreERT2, and Zeb1$^{flox/flox}$ mice [Figure 2a]. A concern that arises from the RT-PCR results is whether the small amount of intact *Zeb1* mRNA would lead to the presence of Zeb1 protein in the corneal endothelium of 4-OHT-injected Zeb1$^{flox/flox}$: UBC-CreERT2 mice. Immunoblotting using proteins isolated from the corneal

endothelium shows that Zeb1 could not be detected in of 4-OHT-injected Zeb1$^{flox/flox}$: UBC-CreERT2 mice whose corneas were treated with FGF2 [Figure 2b]. Intracameral 4-OHT injection and FGF2 treatment induced Zeb1 protein expression in wildtype, UBC-CreERT2, and Zeb1$^{flox/flox}$ mice [Figure 2b].

Our results show that genes in the corneal endothelium can be targeted in a conditional manner. This opens the door for studying adult function of genes in the corneal endothelium that have critical development roles, where knocking out the genes leads to an embryonic or perinatal lethal phenotype. In this report, we demonstrate proof of principle for spatiotemporal gene targeting in the mouse corneal endothelium using an inducible Cre-Lox strategy.

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Conflicts of interest

There are no conflicts of interest.

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