Monoallelic Expression of *Krt12* Gene during Corneal-type Epithelium Differentiation of Limbal Stem Cells

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PURPOSE. The purpose of this study was to characterize a Krt12-Cre knock-in mouse line for corneal epithelium-specific gene ablation and to analyze the allelic selection of the keratin 12 (*Krt12*) gene during corneal type-epithelium differentiation.

METHODS. Knock-in mice were generated by gene targeting. The authors examined the expression patterns of several reporter genes in the corneas of bitransgenic $Krt12^{cre/+}/ROSA^{EGFP}$, $Krt12^{Cre/+}/ZEG$, and $Krt12^{Cre/+}/ZAP$ mouse lines. Krt12 and cre recombinase (Cre) immunostaining was performed. Corneal epithelial cells from bitransgenic $Krt12^{Cre/+}/ROSA^{EGFP}$ mice were examined by fluorescence-activated cell sorter.

RESULTS. Mosaic and spiral expression patterns of EGFP were observed in young and adult bitransgenic $Krt12^{Cre/+}/ZEG$ mice, respectively. Immunostaining revealed that Cre$^-$ cells were also Krt12 negative in the corneal epithelia of $Krt12^{Cre/-}/ZAP$ mice. Using FACS analysis, 60% to 70% of the corneal epithelial cells from $Krt12^{Cre/+}/ROSA^{EGFP}$ mice were EGFP positive, whereas 20% to 30% were negative. RT-PCR revealed that EGFP$^+$ cells express both $Krt12^{Cre}$ and $Krt12^+$ alleles, whereas EGFP$^-$ cells express only $Krt12^+$. In the $Krt12^{Cre/-}$ cornea, the number of epithelial cells expressing Cre is the same as that found in $Krt12^{Cre/Cre}$, which can be explained by the fragility of corneal epithelial cells that did not produce Krt12 because the $Krt12^{Cre}$ allele was not transcribed.

CONCLUSIONS. These observations are consistent with the notion that clonal limbal stem cells randomly activate *Krt12* alleles in the process of terminal differentiation. The authors suggest that this selection is advantageous for retaining epithelial cells expressing the $Krt12^+$ allele and that it allows tolerance to structural mutations of *Krt12*. (*Invest Ophthalmol Vis Sci.* 2010;51:4562–4568) DOI:10.1167/iovs.10-5331

Mammals are mosaic organisms because both alleles of many genes are not ubiquitously or equally expressed by individual cells of the same cell type in a given differentiated tissue. Mosaicism results from monoallelic expression, an unusual form of gene expression in which one allele of a gene is expressed.

When a postzygotic mutation occurs in either the germ line or somatic cells, it leads to an organism with two or more genetically different populations of cells originating from a genetically homozygous zygote. Random X-chromosome inactivation in females was first described by Lyon[1] and is one of the best examples of monoallelic selection. Inheritance of hair color in mammals is another example of mosaic expression patterns of clonal allele activation of melanocytes. For example, many single-gene mutations at a wide variety of mouse pigmentary loci lead to multicolored coats.[2] Those color patterns that are caused by genes expressed directly in melanocytes are clearly clonal patterns reflecting the proliferation and migration of pigment cells[3,4] as they originate from the neural crest.[5] Mintz[2] proposed the phenoclone hypothesis to explain such observations, according to which virtually all mammalian cell types comprise phenotypically different developmental clones, or phenoclones, and virtually all loci have some alleles subjected to such ambiguous *cis*-acting controls. However, there was no other experimental evidence than hair color inheritance derived from melanocytes to substantiate the phenoclone hypothesis.[2] Apart from single-allele activity of genes in cells of X chromosome-linked heterozygotes,[6] the basis for this clonal phenotypic heterogeneity is unknown.

Mosaicism has been used as one mechanism that accounts for segmental skin disease in which the diseased cells are virtually derived from a mutated cell clone at postzygotic stages. In skin disorders, type 1 mosaicism is characterized by segmental lesions according to Blaschko's line, with mutations found in affected tissues but not in healthy tissues. Blaschko's line, named after the German dermatologist Alfred Blaschko, is an invisible skin line thought to trace the migration of embryonic cells.[7] Type 2 mosaicism of autosomal dominant mutation exhibits mild diffused manifestations in most of the body, but linear patterns of exacerbation occur along the Blaschko's line. Recent studies of a patient with Hailey-Hailey disease, also known as familial benign chronic pemphigus, a genetic disorder that causes blisters to form on the skin, demonstrated the loss of heterozygosity of the *ATP2C1* gene in the affected regions of skin.[8,9] However, it remains unclear why those regions of skin that maintain heterozygosity have mild clinical manifestations. One possible explanation is that the mRNA of the mutant allele is unstable and does not lead to the synthesis of a significant amount of mutant protein, causing perturbation of cellular function. Alternatively, the phenomenon can be attributed to clonal activation of an individual allele of the affected gene, which may also lead to the mosaic phenotype in tissue.

In the present study, we examined the possibility of clonal allelic activation taking place within keratinocytes (e.g., corneal epithelial cells). Conventional gene targeting was used to create Krt12-Cre knock-in mice in which an internal ribosome entry site (IRES)-Cre minigene was inserted immediately after the stop codon in exon 8 of the mouse *Krt12* gene. As a result, transcription of the modified

$Krt12^{Cre}$ allele leads to simultaneous synthesis of K12 keratin and Cre recombinase. The expression pattern of Cre was assessed by the expression of reporter genes EGFP and AP during corneal-type epithelial differentiation of limbal stem cells in the corneal epithelium of bitransgenic Krt12-Cre/ROSA-EGFP, Krt12-Cre/ZEG, and Krt12-Cre/ZAP mice, respectively. Our results suggest that clonal activation of individual *Krt12* alleles occurred in limbal stem cells undergoing corneal type-epithelium differentiation.

METHODS

Generation of Krt12-IRES-Cre Knock-in Mice by Gene Targeting

Conventional gene targeting was used to create *Krt12-Cre* knock-in mice. A 3.7-kb *Bam*HI-*Stu*I genomic DNA fragment of *Krt12*, constituting a region extending from intron 2 to the 5′ part of exon 8, was cloned into the *Bam*HI/*Sma*I site of *pBluescriptSKII+* (Stratagene, La Jolla, CA). A 1.1-kb 3′ *Krt12* gene fragment, 3′ part of exon 8 to the 3′ untranslated region, including the poly adenylation signal, was prepared by PCR with the upstream sense primer K12/7152KpnI+, 5′-CGGGGTACCCCGGGCCTCACACGGGCTCCTCTGG, and downstream antisense primer K12/8213AscIKpnI-, 5′-CGGGGTACCCGGTCCGG-GCGCGCCTCAGCTGCTGCCAGGTAGGAGAAAG, and was cloned into the *Kpn*I site of *pBluescriptSKII+*. A minigene, nls-Cre containing nuclear localization signals and the Cre recombinase coding sequence was cloned into the *pIRES* vector (Clontech), and the IRES-Cre *Eco*RI-*Dra*I fragment cloned into *Eco*RI/*Eco*RV of *pBluescriptSKII+*. Neomycin resistance cassette (*NeoR*) was ligated to the *Xho*I site between *IRES-Cre* and the 3′ *Krt12* genomic DNA fragment. Then the phosphoglucokinase diphtheria toxin A (*pgkpr-DTa*) minigene cassette was inserted into the *Not*I site in front of the 3.7-kb *Bam*HI-*Stu*I genomic fragment. The orientation and fidelity of these inserts were verified by enzyme digestion and nucleotide sequence. Fifty micrograms of *Asc*I linearized targeting vector was used for electroporation of R1 embryonic stem (ES) cells. Homologous recombinants were identified by PCR of genomic DNA isolated from neomycin-resistant ES clones for 3′-end recombination with the upstream primer pgk472-, 5′-AAAGCGCAT-GCTCCAGACTGCC, and the downstream primer K12/8431-, 5′-GCAA-CAGAGTTAGGACTTGAACCC. They were verified by PCR of genomic DNA for 5′-end recombination with upstream primer K12/3404+, 5′-GTACTAGGATTACAGACATGGGCCACATAGCCC, and downstream primer IRES37-, 5′-GTAACGTTAGGGGGGGGGGGGAGGGGAGAGGGGCGG, as shown in Figure 1. Recombinant ES cells were used for blastocyst injection with C57BL/6 embryos to obtain germ line chimeras by the transgenic core in the Department of Molecular Genetics, University of Cincinnati. Sibling breeding was used to obtain homozygous Krt12$^{Cre/Cre}$ mice. Genotypes of knock-in mice were identified by PCR with genomic DNA, with primers shown (Fig. 1A) for Krt12Cre and Krt12 wild-type (+) alleles.

Animal Protocol

Krt12-IRES-Cre mice (Krt12-Cre/+) and Krt12 knockout[10] (Krt12$^{-/-}$) mice were crossed with ZEG (*Tg(ACTB-Bgeo/GFP)21Lbe/J*), ROSA-EGFP (*B6;129-Gt(ROSA)26Sortm2Sho/J*), and ZAP (*Tg(ACTB-Bgeo/ALPP)1Lbe/J*) reporter mice (Jackson Laboratory, Bar Harbor, ME).[11–13] All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

LacZ and AP Staining

LacZ Staining. Cryosections prepared from enucleated eyes of 12-week-old mice were fixed in 0.2% glutaraldehyde, 50 mM ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid, and 100 mM MgCl^2 in phosphate-buffered saline (PBS), pH 7.4, for 30 minutes at 4°C. Sections were washed three times for 15 minutes each with LacZ wash buffer (2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in PBS) and then incubated with staining solution containing 0.5 mg/mL X-gal, 4.5 mM potassium ferrocyanide and 4.5 mM potassium ferricyanide in LacZ wash buffer at 25°C for 12 hours.

Alkaline Phosphatase Staining. Cryosections were rinsed in PBS and heated at 70°C for 30 minutes to inactivate endogenous alkaline phosphatases. Alkaline phosphatase was visualized with a staining kit according to the manufacturer's protocol (Fast Red; Sigma-Aldrich, St. Louis, MO).

Histology and Immunostaining

Fifteen-micrometer cryosections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 30 minutes, washed three times for 10 minutes each with PBS, blocked with 5% dry milk in PBS at 4°C overnight. The sections were stained with rabbit polyclonal anti–K12 antibody[14] and anti–Cre antibody (Novagen, San Diego, CA) and were visualized by indirect immunofluorescence with appropriate secondary antibody conjugates (Jackson Laboratory; Molecular Probes, Eugene, OR). Specimens were counterstained with reagent containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Shield; Vector Laboratories, Burlingame, CA). Sections were observed with a confocal laser scanning microscope (LSM 510; Carl Zeiss MicroImaging, Thornwood, NY).

Fluorescence-Activated Cell Sorting and RT-PCR

Enucleated eyes from bitransgenic $Krt12^{+/+}/ROSA^{EGFP}$, $Krt12^{Cre/+}/ROSA^{EGFP}$, $Krt12^{Cre/Cre}/ROSA^{EGFP}$, $Krt12^{Cre/-}/ROSA^{EGFP}$ and $Krt12^{-/-}/ROSA^{EGFP}$ mice were incubated in 1% dispase II (Roche, Indianapolis, IN) in PBS (pH 7.4) at 4°C for 12 hours. The corneal epithelium was peeled from the eye and incubated in 0.25% trypsin-EDTA (Gibco, Carlsbad, CA) at 25°C for 40 minutes and was washed twice in PBS. Fluorescence-activated cell sorting (FACS) was performed (FACSVantage; BD Biosciences, San Jose, CA) with a solid-state laser (Lyt 200 S 488; iCyte, Champaign, IL). Sort gates were set, and the data were analyzed (DIVA software; BD Biosciences). Total RNA was isolated from EGFP$^+$ and EGFP$^-$ cells using reagent (Trizol; Gibco). cDNA was synthesized (AMV Reverse Transcriptase; Promega, Madison WI) and subjected to RT-PCR for detection of Krt12 wild-type and Krt12 IRES-Cre mRNA with the following primer pairs: 5′ common primer Krt12/5202+, 5′-GCTGGGCGTCAAGGCTCGCCTGGAG, and 3′ primer Krt12/7282-, 5′-CAAGACCCAACCTGCATAGAGAATCC, for wild-type Krt12 mRNA; 3′ primer IRES263-, 5′-CGCTACAGACGTTGTTTGT-CTTC, for K12-IRES-Cre mRNA; 5′ primer mGAPDH333+, 5′-GGGTG-GAGCCAAACGGGTCATC, and 3′ primer mGAPDH864-, 5′-GGAGTT-GCTGTTGAAGTCGCAGG, for GAPDH mRNA as control. PCR was performed as follows: 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, and 72°C for 5 minutes. PCR products were analyzed by electrophoresis in 2% agarose gels.

RESULTS

Preparation of Krt12-Cre Knock-in Mouse Line

The knock-in strategy of gene targeting was used to prepare a mouse line, which expresses Cre recombinase under the control of the *Krt12* promoter. The targeting construct contained *IRES-Cre* and *NeoR* minigenes and was used for electroporation of ES cells as shown in Figure 1A. Homologous recombination resulted in the insertion of the minigenes between the stop codon of exon 8 and the polyadenylation signal of the *Krt12* gene. Seven homologous recombinant ES cell clones were obtained (Fig. 1B). Conventional blastocyst injection was used to obtain germ line chimeras. Three chimeras were bred with Swiss black mice to generate heterozygous Krt12-IRES-nls-Cre (Krt12$^{Cre/+}$) knock-in mice. The modified $Krt12^{Cre}$ allele leads to the synthesis of a bicistronic mRNA that allows
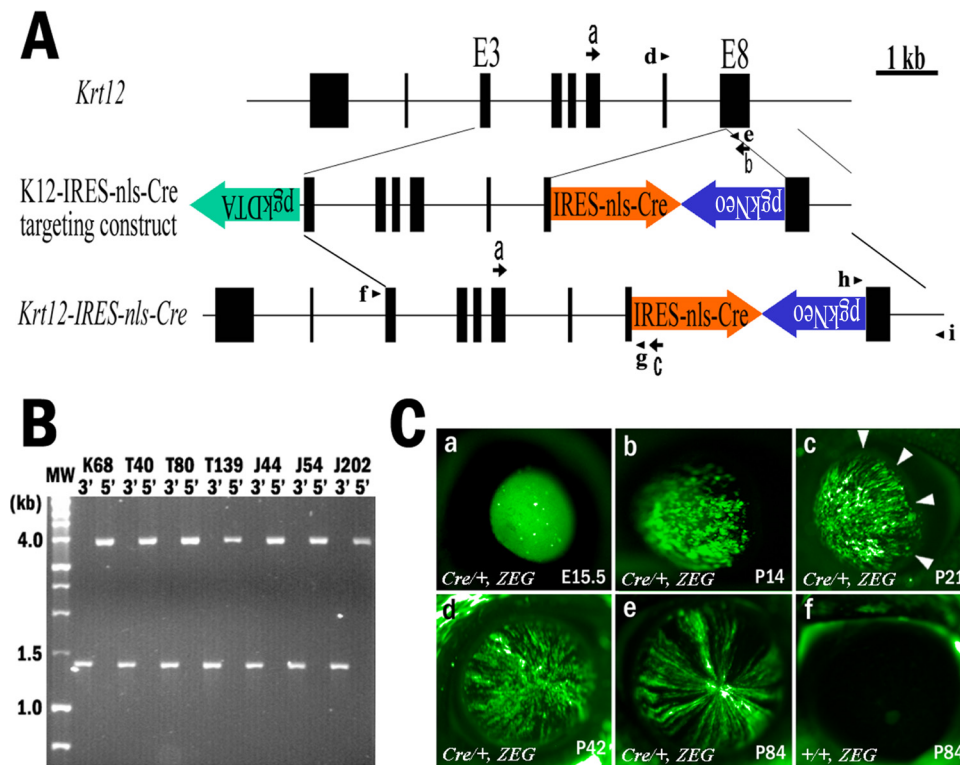
FIGURE 1. Generation of $Krt12^{Cre/+}$ knock-in mice by gene targeting. (**A**) To generate the Krt12-IRES-nls-Cre targeting construct, an IRES-nls-Cre cassette containing IRES, nls, Cre, and SV40-polyA was cloned between the stop codon and the polyadenylation signal in exon 8 of the *Krt12* gene. The pgkNeo cassette (positive selection marker) in a reverse orientation, followed by IRES-Cre and diphtheria toxin A fragment (pgkDTA) cassette (negative selection marker), were placed on the 5′ end of the targeting vector. The *Krt12-IRES-nls-Cre* gene shows the predicted structure of a targeted knock-in allele after homologous recombination. *Arrowheads*: genotyping primer pairs for wild-type (d, e) and for the 5′ (f, g) and 3′ (h, i) ends of *K12-IRES-nls-Cre* gene. *Arrows*: RT-PCR primer pairs for Krt12 wild-type (a, b) and K12-IRES-nls-Cre (a, c). (**B**) Homologous recombinant ES cell clones were identified by PCR, by which the 3′ recombination yields a 1.4-kb product and the 5′ recombination yields a 4-kb product. Locations of primer pairs are indicated in (**A**). (**C**) EGFP expression pattern of $Krt12^{Cre/+}$/ZEG bitransgenic mice (**Ca–e**) during development and maturation. EGFP expression starts from embryonic day (E) 15.5 as a sporadic pattern. After birth, mosaic expression patterns of EGFP are observed throughout the entire corneal epithelium until 2 weeks of age (**Cb**). After 2 weeks, a spiral pattern can be seen invading from the limbus area (*arrowheads*, **Cc**) to the center of the cornea. Mosaic expression patterns gradually disappeared from the center of cornea (**Cd**). After 12 weeks, only spiral expression patterns were observed throughout the entire corneal epithelium (**Ce**). Twelve-week-old Krt12$^{+/+}$/ZEG mouse cornea is shown as negative control (**Cf**). Krt12 knock-in allele (cre); Krt12 wild-type allele (+).

simultaneous synthesis of keratin 12 and Cre recombinase when the modified *Krt12Cre* alleles are transcribed by the corneal epithelial cells of knock-in mice.

K12 Keratin and Reporter Gene Expression Pattern in Corneas of Bitransgenic Mice

To examine the functionality of the knock-in mouse line, $Krt12^{Cre/+}$ mice were crossed with the ZEG reporter mouse line (Jackson Laboratory) to obtain $Krt12^{Cre/+}$/ZEG bitransgenic mice. ZEG mice contain the *LacZ* gene and a stop codon that are flanked by lox P sites followed by the *EGFP* gene. In the absence of Cre, mice under the control of the chicken β-actin promoter will express β-galactosidase but not EGFP. In the presence of Cre, recombination occurs, removing *LacZ* and turning on the expression of EGFP. These mice allow us to identify K12-expressing cells based on the expression of EGFP. Interestingly, mosaic and spiral expression patterns of EGFP were observed in the corneal epithelium of young and adult $Krt12^{Cre/+}$/ZEG bitransgenic mice, respectively (Fig. 1C). One possible explanation for such expression patterns might be

derived from the selected activation of either the $Krt12^+$ wild-type allele or the $Krt12^{Cre}$ knock-in allele by individual limbal stem cell clones undergoing corneal-type epithelial differentiation. Interestingly, sibling breeding of $Krt12^{Cre/+}$/ZEG revealed that Krt^{Cre} and ZEG alleles were always cosegregated and did not yield $Krt12^{Cre/Cre}$/ZEG offspring, suggesting that, like $Krt12$, ZEG is localized on mouse chromosome 11. Thus, $Krt12^{Cre/+}$/ZEG bitransgenic mice are not suitable for further analysis into the clonal activation of $Krt12$ alleles during corneal type-epithelial differentiation. To circumvent this difficulty, knock-in $Krt12^{Cre/+}$ and knockout $Krt12^{-/-}$ mice were crossed with ZAP reporter mice (Jackson Laboratory). The ZAP reporter mouse line works similarly to the ZEG reporter mouse except that EGFP is replaced by alkaline phosphatase. One benefit of using this mouse line is that β-galactosidase and alkaline phosphatase staining can be performed and visualized on a single section. Thus, those corneal epithelial cells expressing Cre were stained red (Fast Red; Sigma-Aldrich), whereas those that were negative for Cre were stained *blue* by X-gal stain because of the synthesis of β-galactosidase. In addition,

immunofluorescence staining for keratin K12 and Cre recombinase and histochemistry of AP and β-galactosidase activities were performed with corneas of 12-week-old $Krt12^{Cre/+}/ZAP$, $Krt12^{Cre/Cre}/ZAP$, $Krt12^{Cre/-}/ZAP$, and $Krt12^{-/-}/ZAP$ mice. As shown in Figure 2 in $Krt12^{Cre/+}/ZAP$ mouse corneas, a spiral pattern was observed for both AP and β-galactosidase (Fig. 2A). Immunostaining with an anti–K12 antibody showed ubiquitous expression of keratin 12 by almost all corneal epithelial cells except for a few basal corneal epithelial cells (progenitor cells) that are still undifferentiated, as had been shown previously.[15] Despite the fact that nearly all cells in the corneal epithelium expressed K12, many corneal epithelial cells were negative for Cre (Figs. 2C-E). These observations are consistent with the notion of clonal activation of one and both of the two *Krt12* alleles in the process of corneal-type epithelial differentiation. This suggestion is further supported by the expression pattern of the AP reporter gene in corneas of $Krt12^{Cre/Cre}/ZAP$ mice in which the expressions of K12 keratin and Cre genes were ubiquitous by all differentiated corneal epithelial cells except basal cells at the limbus and a few undifferentiated cells in the mid-peripheral regions of the cornea (Figs. 2F-J). To further elucidate the hypothesis of clonal activation of *Krt12* alleles, we investigated the expression pattern of the *AP* reporter gene, Cre, and *Krt12* in the corneal epithelium of $Krt12^{Cre/-}/ZAP$ mice in which one *Krt12* allele was ablated.[10] Interestingly, cells expressing K12 also expressed Cre (Figs. 2K-O). The corneal epithelium of $Krt12^{-/-}/ZAP$ mice lacked AP, K12, and Cre, but β-galactosidase was detected in all corneal epithelial cells.

Clonal Activation (Allelic Selection) of $Krt12^{Cre}$ Knock-in and $Krt12^+$ Alleles by Corneal Epithelial Cells *of* $Krt12^{Cre/+}/ROSA^{EGFP}$ Bitransgenic Mice

To further examine the possibility of allelic selection of the *Krt12* allele during corneal-type epithelial differentiation, $Krt12^{Cre/+}$ mice were mated with ROSA-EGFP reporter mice to obtain bitransgenic $Krt12^{Cre/+}/ROSA^{EGFP}$ mice in which Cre expression from the activated $Krt12^{Cre}$ allele leads to EGFP expression after the excision of stop elements within the $ROSA^{EGFP}$ allele of corneal epithelial cells.[12] $Krt12^{Cre/+}/ROSA^{EGFP}$ bitransgenic corneal epithelial cells were sorted by FACS (Fig. 3B). In $Krt12^{Cre/+}/ROSA^{EGFP}$ bitransgenic mouse corneas, 60% of the cells were EGFP positive, whereas 30% were negative. On the other hand, approximately 85% of the corneal epithelial cells from $Krt12^{Cre/-}/ROSA^{EGFP}$ mice were EGFP positive. In $Krt12^{Cre/Cre}/ROSA^{EGFP}$ bitransgenic mouse corneas, 85% to 90% of the corneal epithelial cells were EGFP positive, and 5% were negative (Figs. 3A-E). At 12 weeks of age, not all basal epithelial cells expressed K12, making the percentage of EGFP$^+$ cells <100%. $Krt12^{Cre/-}/ROSA^{EGFP}$ bitransgenic corneas had nearly the same percentage of cells expressing EGFP as did $Krt12^{Cre/Cre}/ROSA^{EGFP}$ corneas. To determine the mRNA expression of each *Krt12* allele (wild-type or knock-in), RT-PCR was performed using total RNA isolated from EGFP$^+$ and EGFP$^-$ cells. Interestingly, EGFP$^+$ cells expressed both the $Krt12^{Cre}$ knock-in allele and the $Krt12^+$ allele, whereas EGFP$^-$ cells expressed only the $Krt12^+$ allele (Fig. 3F).

Based on FACS analysis, it is apparent that there is a small population of cells that do not express EGFP. Several possibil-
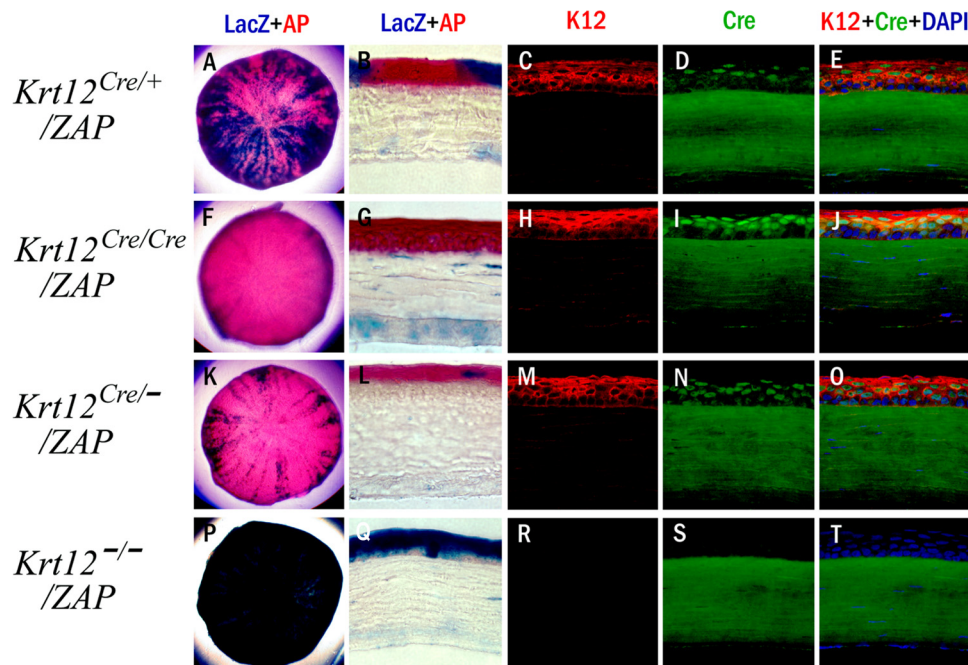

FIGURE 2.   Reporter gene expression. Cre recombinase expression in $K12^{Cre/+}/ZAP$, $K12^{Cre/Cre}/ZAP$, and $K12^{Cre/-}/ZAP$ mouse corneas. Corneas from 12-week-old mice were used for whole mount staining for β-galactosidase (Cre$^-$ cells, *blue*) and AP (Cre$^+$ cells, *red*) (**A, F, K, P**). $K12^{Cre/+}/ZAP$ cornea is stained by a *red* and *blue* spiral pattern. In $K12^{Cre/Cre}/ZAP$ and $K12^{Cre/-}/ZAP$ cornea, most of the epithelium is stained *red* with small slivers of *blue*. Cryosections (**B, G, L, Q**) are stained by β-galactosidase (*blue*) and AP (*red*), and adjacent sections of each genotype are stained by K12 (**C, H, M, R**, *red*) and Cre (**D, I, N, S**, *green*) and then merged with DAPI (**E, J, O, T**). In the $K12^{Cre/+}/ZAP$ cornea, LacZ$^+$ and AP$^+$ cell populations appear side by side, and a serial section shows that most of the epithelium is positive for Krt12. $K12^{-/-}/ZAP$ serves as the negative control, and nonspecific binding of anti–Cre antibody can be observed in the corneal stroma. $Krt12^{Cre}$, Cre knock-in allele; $Krt12^+$, wild-type allele.
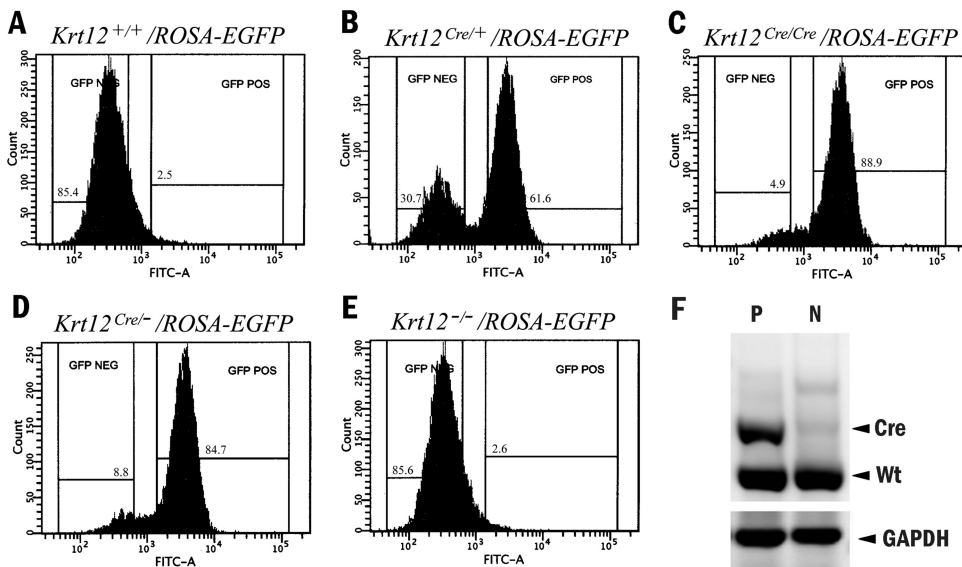
A *Krt12 +/+/ROSA-EGFP*

B *Krt12 Cre/+/ROSA-EGFP*

C *Krt12 Cre/Cre/ROSA-EGFP*

D *Krt12 Cre/-/ROSA-EGFP*

E *Krt12 -/-/ROSA-EGFP*

F

FIGURE 3. FACS of EGFP$^+$ and EGFP$^-$ cells. FACS of *Krt12$^{+/+}$/ROSA^{EGFP}*, *Krt12$^{Cre/+}$/ROSA^{EGFP}*, *Krt12$^{Cre/Cre}$/ROSA^{EGFP}*, *Krt12$^{Cre/-}$/ROSA^{EGFP}*, and *Krt12$^{-/-}$/ROSA^{EGFP}* corneal epithelial cells according to the expression of *ROSA^{EGFP}*, and isolated RNA was subjected to RT-PCR. Corneas from 12-week-old mice of each genotype were sorted based on EGFP fluorescence intensity (**A–E**). Sort gates were set to include the GFP$^+$ (percentages are shown on the horizontal gate lines) cells. (**F**) RT-PCR from a *Krt12$^{Cre/+}$/ROSA^{EGFP}* cornea. GFP$^-$ cells only express mRNA of the *Krt12$^+$* allele, whereas GFP$^+$ cells express mRNA of both the *Krt12^{Cre}* allele and the *Krt12$^+$* allele. *Krt12^{Cre}*, Cre knock-in allele; *Krt12$^+$*, wild-type allele.

ities may explain this EGFP$^-$ cell population. As mentioned earlier, not all basal epithelial cells express K12 at 12 weeks of age because they are not fully differentiated. In addition, it may be that in this population of cells, both *Krt12^{Cre}* alleles were not selected and silenced. To gain some insight into this cell population, 12-week-old *Krt12$^{cre/cre}$/ZAP* mice were stained for β-galactosidase and alkaline phosphatase. β-Galactosidase–positive cells, which are those cells not expressing K12, can be found within the limbus and within the suprabasal and superficial layers of the corneal epithelium in the midperiphery. Interestingly, β-galactosidase–positive cells are not present in the central epithelium (Fig. 4). The suprabasal and superficial location of these β-galactosidase–positive cells suggest, though not conclusively, that these cells may be the result of selecting both silenced alleles. Typically, cells that are not fully differentiated and, therefore, that do not express K12 are restricted to the basal epithelium.

DISCUSSION

Monoallelic expression has been shown to occur in several genes, including T-cell receptor, Toll-like receptor 4, and interleukins, to name a few.[16-20] In this article, we provide evidence to show allelic selection of the *Krt12* gene occurs within the corneal epithelium. Previous studies have shown that corneal epithelial stem cells are located in the basal layer of the limbal epithelium.[21-23] Limbal stem cells divide and centripetally migrate into the cornea to maintain the stratified corneal epithelium. Our previous studies showed that the corneal epithelium-specific *Krt12* gene starts expressing when the limbal epithelium migrates onto the cornea.[24,25] Although our knowledge of gene silencing and activation has increased in the past few years, there remains a large black box in the genetic and epigenetic regulatory mechanisms dictating gene expression. For example, X-chromosome inactivation has been well characterized, but the mechanism behind autosomal gene silencing is still poorly understood except for imprinting of maternal and paternal inheritance.

In this study, our original purpose was to make a corneal epithelium-specific knockout mouse using the Cre-loxP system. Because of the lack of a functional cornea-specific promoter, we chose to use knock-in gene targeting to modify the *Krt12* allele so that it contains an IRES-nls-Cre minigene that allows the synthesis of a bicistronic mRNA for the concurrent synthesis of both Krt12 and Cre recombinase. *Krt12$^{Cre/+}$* showed cornea-specific Cre expression, but 20% to 30% of the

Krt12$^{Cre/Cre}$ 12 weeks LacZ-AP
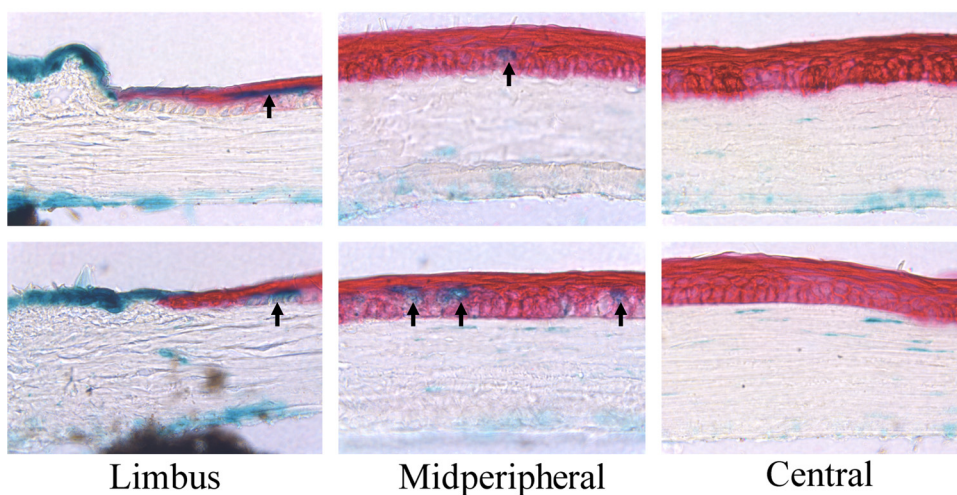

Limbus   Midperipheral   Central

FIGURE 4. Monoallelic selection of *Krt12* alleles. Twelve-week-old *Krt12$^{cre/cre}$/ZAP* mice were stained for β-galactosidase and alkaline phosphatase. The presence of Cre$^-$ cells (*blue*) can be seen in the suprabasal and superficial epithelial layers of limbus and in the midperipheral region of the cornea. No *blue* cells are present in the central cornea.

epithelial cells did not express Cre (Fig. 2). In the $Krt12^{Cre/Cre}$ cornea, more than 85% of the epithelial cells expressed Cre. These results indicate that to successfully knock out genes within the cornea epithelium in a Krt12-dependent fashion, $Krt12^{Cre/Cre}$ mice would have to be used. Interestingly, $Krt12^{Cre/-}$ corneas had a population of Cre$^+$ epithelial cells similar to that of $Krt12^{Cre/Cre}$ mouse corneas. Our previous studies have shown that in $Krt12^{\pm}$ mice, most of the corneal epithelial cells express the K12 protein.[24,25] Our interpretation of these results was simply that the $Krt12^+$ allele was expressed in all the corneal epithelial cells. However, given the data presented here, it is likely that this occurred because some limbal stem cell clones do not express $Krt12^+$ alleles, resulting in Krt12$^-$ cells. The absence of K12 is likely to make these cells fragile and unable to complete their centripetal migration into the central corneal epithelium; however, more research is needed to confirm this hypothesis. In contrast, the cells that express the $K12^+$ allele gain the advantage and become the dominant cell type present in $K12^{\pm}$ corneas. This hypothesis is partially substantiated by the observation that a small number of K12$^-$ suprabasal corneal epithelial cells were found in the limbus and midperipheral cornea of $K12^{Cre/Cre}$/ZAP mice, which did not complete their centripetal migration into the central corneal epithelium (Fig. 4).

Based on the data presented, we propose that allelic selection of the *Krt12* gene occurs within each limbal stem cell clone leading to the generation of four possible outcomes. Figure 5 represents these possibilities and the resultant reporter gene expression within the corneal epithelium for $Krt12^{Cre/Cre}$, $Krt12^{Cre/+}$, and $Krt12^{Cre/-}$ mice. It is clear to see that even with a stem cell containing the $Krt12^{Cre}$ allele, there is a chance that this allele will be untranscribed or silenced. If both alleles are silenced, these cells will fail to express EGFP and will eventually be lost before reaching the central cornea possibly cell because of fragility in the absence of K12 keratin that is essential for the maintenance of corneal epithelial cell integrity.[10] Naturally there exist progenitor cells in the basal layer of the corneal epithelium that do not express Krt12; however, there remains a population of cells that have silenced $Krt12^{Cre}$ alleles and that are not progenitor cells. This suggestion is substantiated by results shown in Figure 4, in which the cells containing both silenced $Krt12^{Cre}$ alleles can be seen in the suprabasal and superficial layers of the midperipheral cornea but are absent from the central cornea because of cell fragility similar to that seen in $Krt12^{Cre/-}$/$ROSA^{EGFP}$ mice (Fig. 3). Alternatively, these undifferentiated Krt12 negative cells in the superficial-midperipheral cornea may finally undergo differentiation and express one or both $Krt12^{Cre}$ alleles toward the end of their journey of centripetal migration.

It should be noted that allelic expression of the *Krt12* gene provides an evolutionary advantage to allow the maintenance of corneal epithelial integrity in those who inherit a heterozygous *Krt12* mutation. Krt12 protein is a member of the building blocks of polymeric corneal epithelial intermediate filaments. As such, if both alleles were equally expressed, a heterozygous mutation would lead to interruption in the formation of functional intermediate filaments in all corneal epithelial cells, rendering severe defects to the corneal epithelium. On the contrary, allelic selection would allow cells that do not express the mutant *Krt12* allele but express the wild-type allele to gain the advantage in their centripetal movement during corneal epithelial cell differentiation. We have also shown that because of the allelic selection of *Krt12*, to successfully ablate genes in the corneal epithelium $Krt12^{cre/cre}$
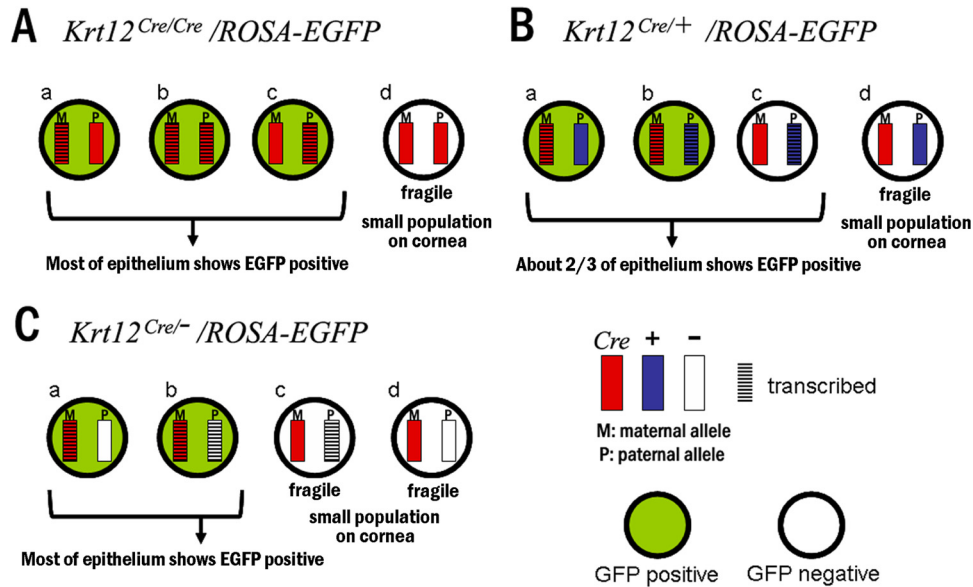

FIGURE 5. Model of allelic selection of the *Krt12* gene. There are four possible patterns of Krt12 expression based on the random event of allelic selection: (*a*) maternal allele only, (*c*) paternal allele only, (*b*) both alleles, (*d*) neither allele. Each limbal stem cell selects one of them. (**A**) In the $Krt12^{Cre/Cre}$/$ROSA^{EGFP}$ bitransgenic mouse cornea, if the stem cell selects pattern *d*, this cell lineage will not express Krt12 protein and will become fragile and eventually lost. Therefore, if pattern *d* is chosen, that cell lineage will have a competitive disadvantage in terms of survival compared with patterns *a*, *b*, and *c*. These three selections will express EGFP throughout nearly all the corneal epithelium. (**B**) In the $Krt12^{Cre/+}$/$ROSA^{EGFP}$ bitransgenic mouse cornea, only cell lineage *d* fails to express Krt12. Patterns *a* and *b* express EGFP, but pattern *c* does not; therefore, approximately two-thirds of the epithelial cells express EGFP. (**C**) In the $Krt12^{Cre/-}$/$ROSA^{EGFP}$ bitransgenic mouse cornea, patterns *a* and *b* express both Krt12 and EGFP, but patterns *c* and *d* do not have Krt12 protein. Therefore, most of the corneal epithelium will become EGFP positive. It should be noted that the ratio of transcriptional activation for each allele is not necessarily 50%. $Krt12^{Cre}$, Cre knock-in allele; $Krt12^+$, wild-type allele.

mice have to be used. Although we show the allelic selection of *Krt12*, the genetic or epigenetic mechanism responsible for such selection remains unclear and will be pursued in the future.

Acknowledgments

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