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Mastermind-like transcriptional co-activator-mediated Notch signaling is indispensable for maintaining conjunctival epithelial identity

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SUMMARY

Conjunctival goblet cells primarily synthesize mucins to lubricate the ocular surface, which is essential for normal vision. Notch signaling has been known to associate with goblet cell differentiation in intestinal and respiratory tracts, but its function in ocular surface has yet to be fully characterized. Herein, we demonstrate that conditional inhibition of canonical Notch signaling by expressing dominant negative mastermind-like 1 (dnMaml1) in ocular surface epithelia resulted in complete suppression of goblet cell differentiation during and subsequent to development. When compared with the ocular surface of wild-type mice (OS^{Wt}), expression of dnMaml1 at the ocular surface ($OS^{dnMaml1}$) caused conjunctival epithelial hyperplasia, aberrant desquamation, failure of Mucin 5ac (Muc5ac) synthesis, subconjunctival inflammation and epidermal metaplasia in cornea. In addition, conditional deletion of *Notch1* from the ocular surface epithelia partially recapitulated $OS^{dnMaml1}$ phenotypes. We have demonstrated that N1-ICD (Notch1 intracellular domain) transactivated the mouse Krüppel-like factor 4 (*Klf*) promoter and that Klf4 directly bound to and significantly potentiated the *Muc5ac* promoter. By contrast, $OS^{dnMaml1}$ dampened Klf4 and Klf5 expression, and diminished Muc5ac synthesis. Collectively, these findings indicated that Maml-mediated Notch signaling plays a pivotal role in the initiation and maintenance of goblet cell differentiation for normal ocular surface morphogenesis and homeostasis through regulation of *Klf4* and *Klf5*.

KEY WORDS: Goblet cell, Klf4/5, Notch signaling, Cell fate, Differentiation, Ocular surface, Mouse

INTRODUCTION

Conjunctival goblet cells are polarized epithelial cells primarily distributed at the fornix of the conjunctival epithelium. They secrete aqueous mucins to protect the ocular surface from dryness and other deleterious conditions. Abnormalities of goblet cell mucous secretion invariably link to multiple pathologies of ocular surface disorders such as squamous metaplasia, a hallmark of different forms of dry eye syndrome (De Paiva et al., 2007; Corrales et al., 2011), neurotrophic keratitis (Gilbard and Rossi, 1990), atopy and seasonal ocular allergy (Mantelli and Argüeso, 2008). However, the cellular mechanism underlying goblet cell formation and related pathogenesis of keratoconjunctivitis sicca (KCS or dry eye) is poorly understood.

Notch signaling regulates cell fate decision and plays many crucial roles in controlling goblet cell differentiation in the gut epithelium (Radtke and Clevers, 2005). It has also been suggested that Notch activation contributes to the maintenance of the stratified and non-keratinized corneal (Ma et al., 2007; Vauclair et al., 2007; Djalilian et al., 2008; Nakamura et al., 2008) and conjunctival type epithelial differentiation (Fukushima et al., 2008; Mantelli et al., 2009; Xiong et al., 2011). Notch signaling is a central mediator of short-range intercellular communication in metazoans (Kovall and Blacklow, 2010; Andersson et al., 2011). The Notch receptor consists of a large ectodomain and a membrane-tethered intracellular domain that is derived from a proteolytic cleavage of the Notch precursor protein. Notch ligands, such as those in the Delta-like (Dll1, Dll3 and Dll4) and Jagged (Jag1 and Jag2) families, interact with receptors of the Notch family (Notch1-4) of an adjacent cell. This ligand-receptor interaction induces further proteolytic cleavage of Notch, which leads to the release the Notch intracellular domain (NICD) from the cell membrane (Kopan and Ilagan, 2009). The NICD translocates into the nucleus, where it displaces a histone deacetylase (HDAc) and binds the recombination signal binding protein for immunoglobulin Jκ region (RBP-Jκ) protein of the co-repressor (CoR) complex. It further recruits histone acetyltransferases (HAc) for the formation of an activation complex consisting of Maml (Wu et al., 2000), NICD, RBP-Jκ and HAc, and subsequent transcriptional activation of Notch target genes such as the basic helix-loop-helix transcription factor genes *Hes* and *Hey*, and other genes (Bray, 2006; Gridley, 2007).

Microarray analysis of mRNAs concerning dry eye versus normal conjunctival epithelia demonstrate that members of the canonical Notch signaling pathway, i.e. Notch1, Notch 2, Notch 3, Jagged1 and Delta1, are significantly downregulated (Mantelli et al., 2009), albeit the specific function of the canonical Notch signaling pathway in ocular surface goblet cell differentiation remains largely unknown. We hypothesize that Notch signaling plays a pivotal role in the differentiation and maintenance of goblet cells in order to establish the conjunctival epithelial integrity necessary for ocular surface health. In this study, we have examined Notch loss-of-function phenotypes in transgenic mice that conditionally overexpress dnMaml1 in *Krt14* (K14)-positive cells in the process of ocular surface epithelial morphogenesis during development and in the adult. Ablation of Notch signaling

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by dnMaml1 resulted in the failure of goblet cell formation during development and the loss of pre-existing goblet cells in the adult, thus producing symptoms resembling KCS. We investigated the pathogenesis of conjunctival and corneal squamous metaplasia in this transgenic mouse model and found that Notch signaling augmented the expression of Klf4 and Klf5, resulting in goblet cell differentiation by upregulating transcription of Muc5ac and downregulating conjunctival epithelial proliferation. Therefore, suppression of the Notch canonical pathway can abrogate normal goblet cell differentiation and lead to KCS with mucin deficiency.

MATERIALS AND METHODS

Mouse strains and genotyping

The *Rosa*$^{LSL\text{-}dnMaml1}$ mouse model, in which dnMaml1 is synthesized upon the excision of the *LSL* (*Loxp-Stop-Loxp*) element in cells expressing Cre recombinase, has been reported previously (Tu et al., 2005). The dnMaml1 is a 62 amino acid peptide of the N-terminal basic domain (BD) of Maml1 that is capable of interacting with NICD, but lacks the p300 transactivation domain (TAD) necessary to bind HAc (histone acetyl transferase). Therefore, dnMaml1 is a pan-Notch inhibitor that interferes with the endogenous function of Maml proteins and inhibits the activation of all four Notch receptors (Weng et al., 2003). Other transgenic mouse lines, including *K14-rtTA* (stock number 008099) (Nguyen et al., 2006b), *tetO-Cre* (*TC*) (stock number 006224) (Perl et al., 2002), *N1IP-Cre* (stock number 006953) (Vooijs et al., 2007), *Rosa*$^{mTmG/mTmG}$ (stock number 007576) (Muzumdar et al., 2007) and *Notch1*$^{flox/flox}$ (stock number 006951) (Yang et al., 2004), were purchased from the Jackson Laboratory (Bar Harbor, MN, USA). Compound transgenic mice were generated via mating. All the mice were bred at the Animal Facility of the University of Cincinnati Medical Center. Experimental procedures for handling the mice conformed to the statement for use of experimental animals in ophthalmology and visual science of ARVO (Association for Research in Vision and Ophthalmology) and were approved by the Institutional Animal Care and Use Committee, University of Cincinnati/College of Medicine. The identification of each transgene allele was performed by PCR genotyping with tail DNA.

Administration of Dox chow

To induce dnMaml1 expression, compound transgenic mice (older than P21) were fed with Dox-chow (doxycycline 1 g/kg chow, Bioserv, Frenchtown, NJ) *ad libitum*. Neonates were administrated with Dox at birth (P0) by feeding the nursing mother with Dox chow *ad libitum*. dnMaml1 expression is persistent and irreversible upon Dox induction in K14-positive cells and in the progeny of the *K14-rtTA/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple transgenic mouse strain. Thus, the canonical Notch signal pathway should be suppressed in K14-positive cells and their progeny. Control animals were littermates with either single or double transgene(s).

Histological analysis

Mouse samples were fixed overnight in 4% PFA/PBS [paraformaldehyde in 1× phosphate-buffered saline (pH 7.2)] and then embedded in OCT compound or paraffin blocks. For frozen OCT embedded blocks, *N1IP-Cre/Rosa*$^{mTmG/Wt}$ eye tissue sections (10 μm) were counterstained with 1 μg/ml DAPI (4,6-diamidino-2-phenylindole) in PBS. For paraffin wax-embedded tissue blocks, deparaffined sections (5 μm) were stained with Hematoxylin and Eosin or periodic acid Schiff (PAS).

Detection of cell proliferation

For detection of cell proliferation, BrdU (Sigma, 80 μg/g body weight) was injected intraperitoneally into the experimental mice 2 hours prior to sacrificing. Ocular tissues were then dissected, fixed in 4% PFA/PBS, dehydrated through graded alcohols, embedded in paraffin wax and sagittal sections (5 μm) were treated with 3 N HCl for 15 minutes at room temperature followed by three washes in PBS. Immunodetection of BrdU was performed using mouse anti-BrdU monoclonal antibody followed by Alexa 555-labeled rabbit anti-mouse IgG and the sections were counterstained with DAPI. The total number of cell nuclei (DAPI-positive), as well as the number of BrdU-labeled nuclei on sections were counted and recorded from five continuous sections. The cell proliferation index was calculated as percentage of the cell nuclei with BrdU labeling.

Immunofluorescence stainings

Tissue sections (5 μm) were subjected to antigen retrieval by immersing slides in sodium citrate buffer [10 mM sodium citrate, 0.05% Tween 20 (pH 6.0)] preheated to boiling and allowed to stand at room temperature for 30 minutes. Tissue sections were then blocked with 3% BSA in PBS containing 0.05% NP-40 for 1 hour at room temperature, then incubated overnight at 4°C with the primary antibodies diluted in the same buffer as described in supplementary material Table S1. After three washes in PBST (PBS/0.1% Tween 20), slides were incubated at room temperature for 1 hour with the Alexa 488- or Alexa 555-conjugated secondary antibodies (Invitrogen) and 1 μg/ml DAPI as a nuclear counterstain, washed with PBST again and mounted with Mowiol (Sanofi-Aventis US). Sections were examined and photographed using a Zeiss microscope equipped with a camera (Axiocam Mrm) (Carl Zeiss). For data acquisition, we used the Axiovision 4.6 software (Carl Zeiss).

Chromatin immunoprecipitation (ChIP) assay

Mouse NIH3T3 and NCI-H292 (Banks-Schlegel et al., 1985) (ATCC: CRL-1848) cell lines were transfected with 20 μg of *pLIA-mNIC-myc* plasmid (Warren et al., 2010) (Addgene plasmid 15131) and *pcDNA3.3-KLF4* cDNA (Bao and Cepko, 1997) (Addgene plasmid 26815) using GeneJammer transfection reagent (Invitrogen). Forty-eight hours post-transfection, cells were cross-fixed with 1% formaldehyde at 37°C for 10 minutes and subjected to ChIP assay with antibodies against RBP-Jκ (Abcam, Cat#ab25949) and Klf4 (R&D, Cat#AF3640), respectively, using the ChIP assay kit (Millipore, Cat#17-295) following the manufacturer's instructions. DNAs purified after ChIP were used as templates for PCR to verify the interaction between DNA and protein. PCR primer sets are listed in supplementary material Table S2.

Real-time quantitative PCR (RT-qPCR)

Total RNA (10 μg) was isolated from ocular surface tissues of postnatal day (P) 9 mice that included conjunctiva and cornea, then annealed to random primer and reverse transcribed with avian reverse transcriptase kits (Promega) according to the manufacturer's instructions. cDNA was subjected to PCR using specific primer pairs listed in supplementary material Table S2. RT-qPCR was performed by using the CFX96 real-time system equipped on a C1000 Thermal Cycler (Bio-Rad Laboratories): 1 cycle of 95°C for 3 minutes, then 40 cycles of 95°C for 15 seconds, 62°C for 15 seconds and 72°C for 20 seconds. The cycle threshold values were used to calculate the normalized expression of genes of interest against *Gapdh* using Q-Gene software.

Promoter-luciferase assay

HEK293 cells seeded in six-well plates at 80% confluence were transiently transfected with a mixture of three different types of plasmids: (1) *prhL-TK* (Promega) served as a transfection efficiency control; (2) *pGL3.0-Basic* or recombinant plasmids harboring the mouse *Klf4* promoter (*pGL3.0-mKlf4pr*) (Zheng et al., 2009) or the human *Muc5A/C* promoter (*pGL3.0-Muc5A/Cpr*) (Chung et al., 2009); (3) cDNA expression plasmids *pLIA-mNIC-myc* or *pcDNA3.1-mKlf4* at different doses as indicated. At 48 hours post-transfection, cells were collected with 1× passive lysis buffer (Promega), and luciferase assays were conducted using the Dual Luciferase Reporter (DLR) Assay System (Promega) according to manufacturer's recommendations. Luminescence was measured using a glomax multidetection system (Promega).

Phenol red thread test

A phenol red thread tear test (ZONE-QUICK, Showa Yakuhin Kako) was used to measure mouse tear volume in this study. The thread is originally yellow and turns pink when it comes into contact with tears. Mice were anaesthetized with ketamine and xylazine. Forceps were used to insert the 3 mm folded region of the thread into the palpebral conjunctiva of the eye one-third of the distance from the lateral canthus of the lower eye lid. After 15 minutes, the thread was removed and the tear-stained (pink) region was measured.

Statistical analysis

A two-tailed Student's *t*-test (Excel, Microsoft, Redmond, WA, USA) was used in the analysis of the percentage of PCNA-, ΔNp63-, Pax6-, BrdU- or Klf4-positive and TUNEL-positive cells. All quantification data are presented as mean±s.e.m. Student's *t*-test was used to analyze the significance of difference; $P<0.05$ was considered statistically significant.

RESULTS

Kinetics of Notch1 activation during mouse eye development

Notch signaling plays a pivotal role in cell differentiation in a wide variety of tissues. However, the Notch activation patterns in the ocular surface remain largely unknown. To fill the gap of the lack of knowledge regarding the function of Notch1 in the eye, it is imperative to determine the spatiotemporal pattern of Notch activation during eye development. To achieve this objective, we crossed two transgenic knock-in mouse lines, i.e. *N1IP-Cre* (Vooijs et al., 2007) and *Rosa*$^{mTmG/mTmG}$ (Muzumdar et al., 2007), to generate a double-transgenic *N1IP-Cre/Rosa*$^{mTmG/Wt}$ mice, which allowed us to trace the progeny of cells that have undergone moderate to strong Notch1 activation during development. In ocular tissues of the *N1IP-Cre*/*Rosa*$^{mTmG/Wt}$ double-transgenic mice, there was very little Notch1 activation in the lens at E15 (embryonic day 15), P1 (postnatal day1) and P30 (Fig. 1A-C). Notch1 activation was mainly present in eyelid, conjunctiva and limbus/cornea epithelium at E15 (Fig. 1A) and P1 (Fig. 1B). However, at P30, cells that had undergone Notch1 activation were found predominantly in the neural retina and retinal pigment epithelium, but relatively fewer were in epidermis (Fig. 1C,C‴). Most interestingly, the distribution of Notch1 activated cells in the ocular surface was preferentially restricted to the conjunctiva (Fig. 1C,C′), but absent in the limbus/cornea (Fig. 1C,C″). These results strongly suggest that the Notch signaling pathway may positively regulate goblet cell differentiation in conjunctival epithelium and prompted us to investigate Notch function in ocular surface morphogenesis.

Pathogenesis of ocular surface tissues due to abrogation of Notch signaling by dnMaml1

To examine the role of Notch signaling in ocular surface epithelia, we created a loss-of-function model by crossing *Rosa*$^{LSL\text{-}dnMaml1}$ (Tu et al., 2005) and *K14-rtTA/TC* mouse lines so that dnMaml1 would be expressed in all K14-positive basal keratinocytes of the *K14-rtTA/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple-transgenic offspring upon Dox induction (Fig. 2A). Neonatal pups were administrated with Dox at birth (P0) and total *Maml1* mRNA levels, including full-length and *dnMaml1*, were measured by RT-qPCR. Fig. 2B indicates that total *Maml1* expression levels detected from the ocular surface tissues of *K14-rtTA/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple-transgenic mice increased 19.8-fold when compared with those of *TC/Rosa*$^{LSL\text{-}dnMaml1}$ double-transgenic mice. Immonofluorescence staining for GFP, which is indicative of expression of the dnMaml1-GFP fusion protein, showed specific GFP expression (red fluorescence) in the conjunctival and corneal epithelia in OS^{dnMaml1} mice (Fig. 2C″,C‴) but not in OS^{Wt} littermates (Fig. 2C,C′). To determine whether the expression of dnMaml1 could influence canonical Notch target gene expression, we examined *Hey1* and *Hes1* expression, and

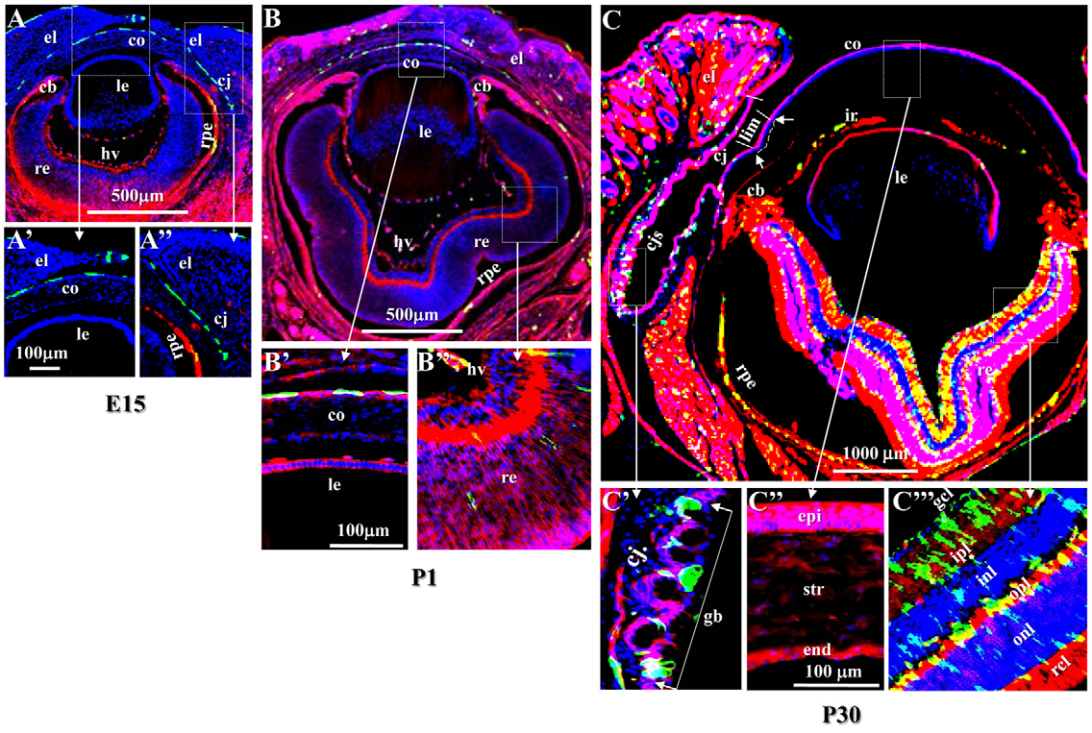


Fig. 1. *In vivo* mapping of Notch1 activation during mouse eye development. (**A-C‴**) Fluorescent micrographs were taken from frozen sections (10 μm) of *N1IP-Cre*^{low}/*Rosa*$^{mTmG/Wt}$ double transgenic mouse at embryonic day 15 (E15) (A-A″), P1 (B-B″) and P30 (C-C″). EGFP-positive (green) signals represented cells in which Notch1 signaling was activated under physiological conditions. Notch1 signal was particularly stronger in ocular surface epithelia compared with other eye tissues during embryonic (A) and neonatal (B) stages, but became more evident in neural retina and retinal pigment epithelium at P30 (C,C‴). Notch1 activation was restricted to the bulbar conjunctival epithelium (C′) but absent in corneal epithelium (C″). cb, ciliary body; cj, conjunctiva; cjs, conjunctival sac; co, cornea; el, eyelid; gb, goblet cells; hv, hyloid vessel; ir, iris; le, lens; end, endothelium; epi, epithelium; gcl, ganglionic cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; lim, limbus; onl, outer nuclear layer; opl, outer plexiform layer; rcl, rod and cone layer; re, retina; rpe, retinal pigment epithelium; str, stroma.

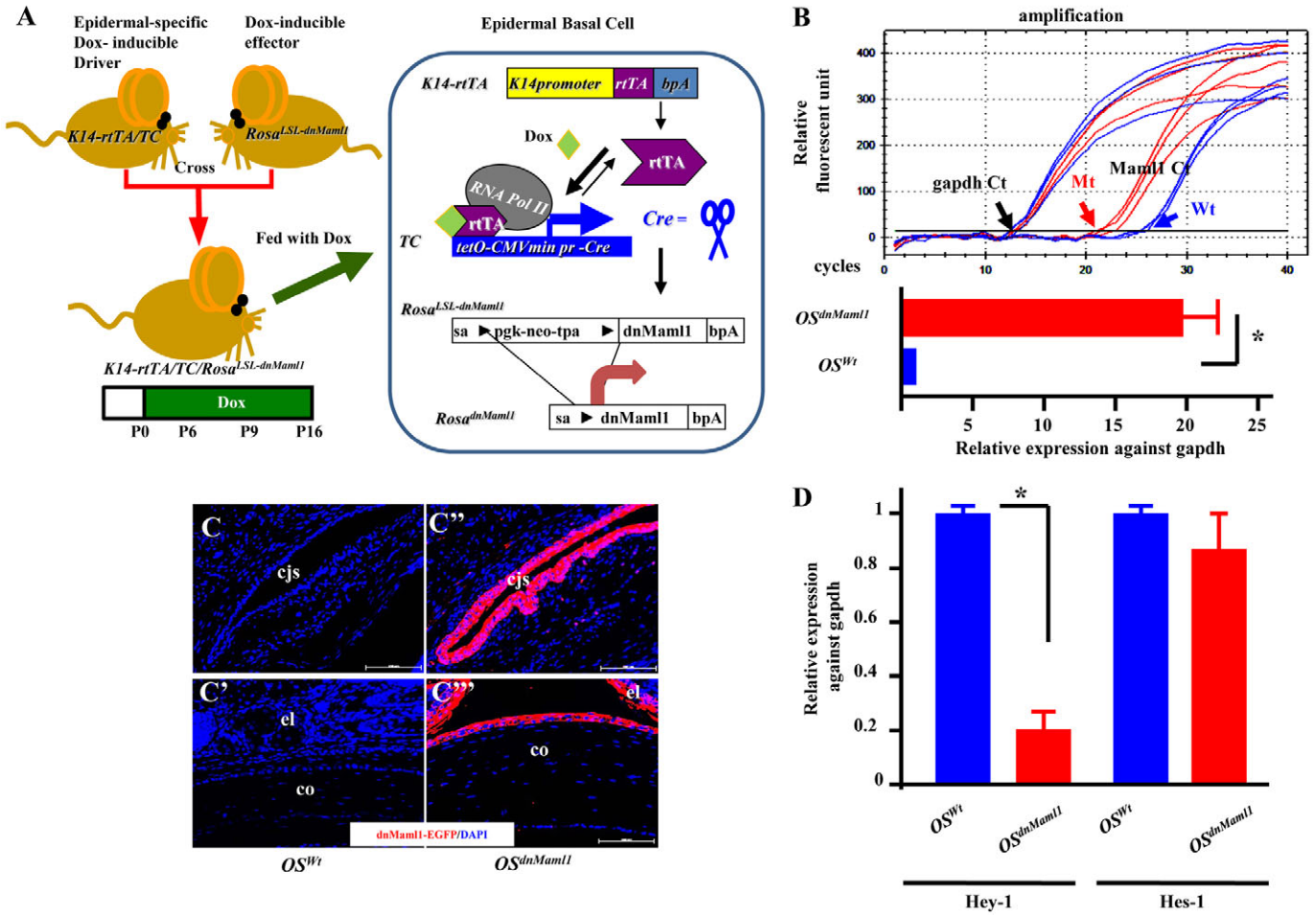


Fig. 2. Conditional misexpression of dnMaml1-inactivated Notch signaling. (**A**) Dox-dependent expression of dnMaml1. A double transgenic mouse, *K14-rtTA/TC*, served as an epidermal-specific Dox-inducible driver. the *Rosa*^{dnMaml1} mouse line served as a Dox-inducible effector. Intercross between *K14-rtTA/TC* and *Rosa*^{dnMaml1} generated triple-transgenic mice *K14-rtTA/TC/Rosa*^{dnMaml1}. Administration of Dox resulted in the expression of *dnMaml1* only in K14-positive cells of the basal layer of all stratified epithelial tissues, including skin and ocular surface tissues. (**B**) RT-qPCR analysis revealed that dnMaml1 mRNA levels were elevated by almost 20-fold upon Dox induction. (**C-C‴**) Immunofluorescent staining of GFP. The positive signals (red) indicate the expression of dnMaml1-GFP fusion protein in conjunctival (C,C′) and corneal (C″,C‴) epithelia. (**D**) As a consequence, Hey1 expression was downregulated by 80% in OS^{dnMaml1}; however, Hes1 expression was not changed significantly. cjs, conjunctival sac; co, cornea; el, eyelid. *P<0.05. Data are mean±s.e.m.

found *Hey1* reduced by ~80% in OS^{dnMaml1} when compared with OS^{Wt} littermate controls. However, *Hes1* expression level exhibited no significant change (Fig. 2D). Histological analysis showed that overexpression of dnMaml1 in K14-positive cells recapitulated epidermal phenotypes observed previously in mice with conditional deletions of RBP-Jκ or *Notch1* by K14-Cre or Tgfb3-Cre (data not shown) (Vauclair et al., 2005; Demehri and Kopan, 2009; Lin et al., 2011). As ocular surface epithelia, including conjunctiva, limbus and cornea, originate from the same part of the surface ectoderm as skin, expression of dnMaml1 should also affect their morphogenesis and maintenance. Indeed, wild-type littermates exhibited clear conjunctival sacs at P9 (Fig. 3A,A′), whereas a mass of cell debris was abnormally present in the conjunctival sac of OS^{dnMaml1} mice (Fig. 3B,B′), indicating significant alterations in early ocular surface morphogenesis. At higher magnifications, the wild-type cells were typically arranged into two layers of epithelia (supplementary material Fig. S1A), while the epithelial cells of OS^{dnMaml1} mice were hyperplastic and aberrantly desquamated from the conjunctiva (supplementary material Fig. S1C). Immunofluorescence staining showed that the desquamated cells within the conjunctival sac were positive for K13, a marker of conjunctival epithelial cells (Fig. 3F), and K14, a marker of basal cells of all stratified epithelia (Fig. 3H), but negative for K12, a marker of corneal type epithelial differentiation (Fig. 3D). These data suggest that OS^{dnMaml1} preferentially and aberrantly trigger conjunctival, but not corneal, epithelial cell desquamation.

Abrogation of Notch signaling by dnMaml1 promoted conjunctiva epithelial cell proliferation

The cellular response to Notch signaling is exquisitely context- and dose-dependent (Nickoloff et al., 2003). To elucidate how Notch function in conjunctival epithelial cell proliferation, expression patterns of endogenous PCNA and ΔNp63 (Fig. 4) and direct measurement of BrdU uptake (supplementary material Fig. S1) were examined by immunofluorescence staining. Our data showed that cells positive for PCNA (Fig. 4B) and ΔNp63 (Fig. 4D) in conjunctival epithelia of OS^{dnMaml1} mice increased 11-fold and 1.8-fold, respectively, when compared to OS^{Wt} littermates (Fig. 4A,C). Likewise, cells positive for BrdU in OS^{dnMaml1} were 14.1-fold more numerous than those in OS^{Wt} littermates (supplementary material

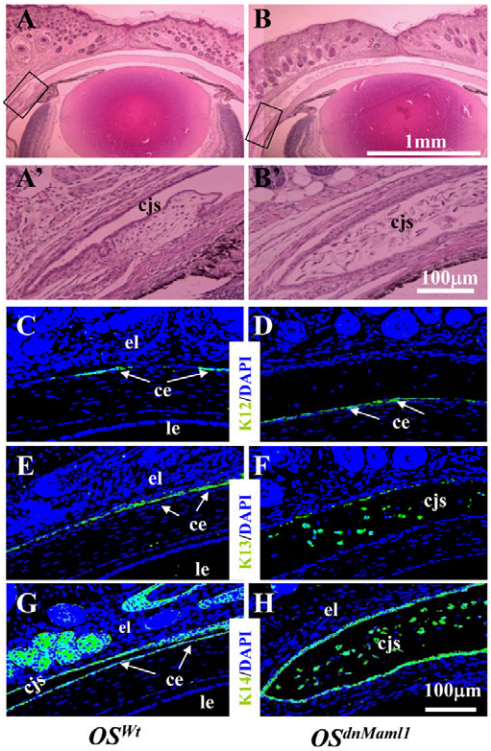


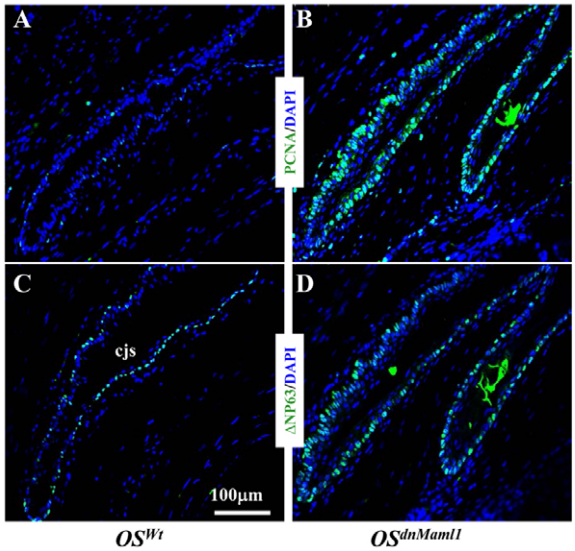
Fig. 3. Misexpression of dnMaml1 in ocular surface epithelia ($OS^{dnMaml1}$) caused aberrant conjunctival epithelial desquamation. (**A-B′**) Hematoxylin and Eosin staining of ocular surface epithelia at P9. Unlike wild-type ocular surface (OS^{wt}), which revealed clearance of conjunctival sac (A; A′ shows inset in A), $OS^{dnMaml1}$ resulted in epithelial cells desquamating into conjunctival sac (B; B′ shows inset in B). (**C-H**) Immunofluorescent staining showed that cells desquamating into conjunctival sac were negative for K12 (C,D) but positive for K13 (F) and K14 (H). (E,G) Normal expression patterns of K13 and K14 in wild-type littermate. ce, cornea epithelium; cjs, conjunctival sac; el, eyelid; le, lens.

Fig. S2E). These results suggest that inactivation of Notch signaling is associated with conjunctival epithelial hyperproliferation, which is consistent with the notion that Notch behaves as a tumor suppressor in epidermal tissues, such as skin (Nicolas et al., 2003).

dnMaml1 abolished conjunctival goblet cell differentiation leading to epidermal metaplasia in cornea

As $OS^{dnMaml1}$ mice preferentially manifested abnormal phenotypes in palpebral, forniceal and bulbar conjunctival epithelia in which goblet cells reside, we examined whether expression of dnMaml1 could affect the differentiation of conjunctival precursor/stem cells into goblet cells. PAS-staining revealed that, in normal mice, mucin-rich goblet cells emerged from conjunctival epithelium around P7-P9, increased in number and formed goblet cell clusters between the second and third week of postnatal development (supplementary material Fig. S3). By contrast, $OS^{dnMaml1}$ mice rendered no sign of goblet cell differentiation at P9 (compare Fig. 5C and 5A) or P16 (compare Fig. 5D and 5B). As expected, immunofluorescence staining of Muc5ac showed that no positive signal was detected in the conjunctiva of $OS^{dnMaml1}$ mice (Fig. 5G), but strong positive signals were observed in the palpebral, forniceal and bulbar conjunctiva of OS^{Wt} littermates (Fig. 5E). In human dry eye syndrome, the reduction of conjunctival goblet cell density is often accompanied with inflammation in ocular surface tissues (Kunert et al., 2001; Argüeso et al., 2002; Gipson and Argüeso, 2003; Ueta et al., 2005). However, we did not detect inflammation in $OS^{dnMaml1}$ mice at P9 (data not shown), but a profound $CD45^{+}$ leukocyte infiltration was found in the sub-conjunctival stroma 3 to 4 days after eyelid opening, at P16 (Fig. 5H), when compared with that of OS^{Wt} littermates (Fig. 5F). These data indicate that the failure of goblet cell formation precedes the subconjunctival inflammation seen in $OS^{dnMaml1}$ mice.

To examine other phenotypic changes in ocular surface tissues following the impairment of goblet cell formation, we examined K12 expression, and found that it was unaltered at P9 (Fig. 3D). However, K12 expression was completely lost from the cornea in $OS^{dnMaml1}$ mice at P16 (compare Fig. 6B with 6A). In addition, we established that paired box homeotic gene 6 (Pax-6), which is known to regulate K12 expression (Shiraishi et al., 1998; Liu et al., 1999), was drastically downregulated in ocular surface epithelia but not in the neural ectoderm-derived ciliary body (compare Fig. 6D with 6C). Similarly, the presence of dnMaml1 also altered K13 and K15 expression patterns in conjunctiva (compare Fig. 6F with 6E, and Fig. 6H with 6G). Most strikingly, at P9 the epidermal differentiation-specific K10 expression pattern was restricted at the mucocutaneous junction of eyelids in both wild-type and $OS^{dnMaml1}$



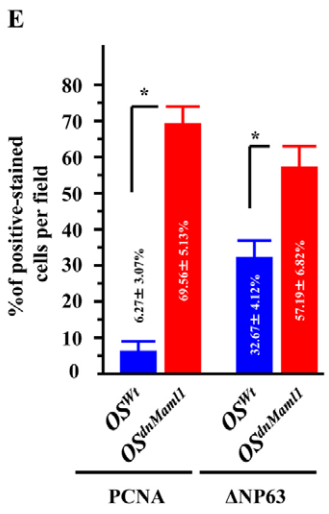


Fig. 4. Expression of dnMaml1 changed cell proliferative activity. (**A-D**) Immunofluorescent micrographs of conjunctival sac from P9 mice probed with anti-PCNA (A,B) or anti-ΔNp63 (C,D) antibody. Both PCNA- and ΔNp63-positive cells were dramatically increased in number in $OS^{dnMaml1}$ (compare B,D with A,C). (**E**) Histogram representation of images of A-D showed significant increase of PCNA and ΔNp63 in $OS^{dnMaml1}$. cjs, conjunctival sac. *P<0.05. Data are mean±s.e.m.

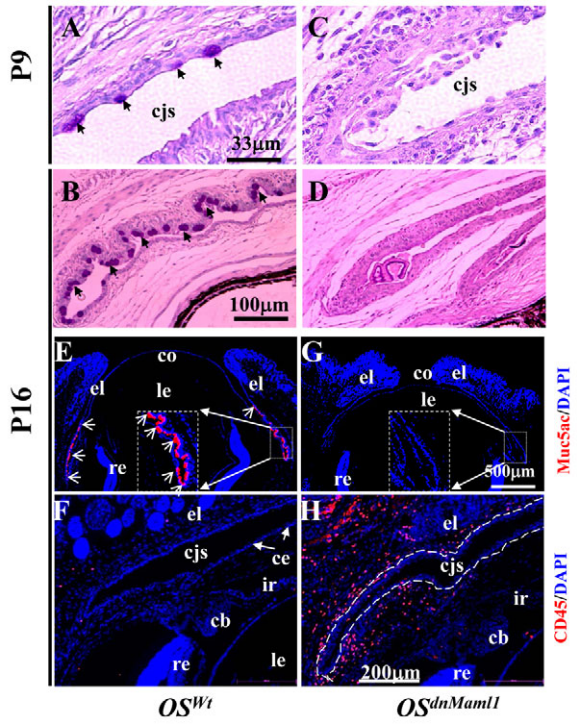


Fig. 5. Expression of dnMaml1 inhibited conjunctival goblet cell differentiation and induced inflammation. (**A**,**B**) Periodic acid Schiff's (PAS) and Hematoxylin staining showed that goblet cells (arrows) became evident at P9 (A) and formed clusters at P16 (B) during postnatal eye development in mice. (**C**,**D**) However, $OS^{dnMaml1}$ revealed hyperplasia in conjunctival epithelium and failed to form goblet cells. (**E**,**G**) Conjunctival goblet cells were identified by positive signals (arrows) using anti-Muc5ac antibody. Most $Muc5ac^{+}$ goblet cells clustered around forniceal and bulbar conjunctival region in P16 (E). By contrast, the expression of dnMaml1 led to total absence of $Muc5ac^{+}$ goblet cells in the same region (G). (**F**,**H**) In addition, very few $CD45^{+}$leukocytes were detected in the naïve ocular surface (F). By contrast, the expression of dnMaml1 led to abundant $CD45^{+}$ leukocyte infiltration into the subconjunctival space (H). cb, ciliary body; cjs, conjunctival sac; co, cornea; el, eyelid; ir, iris; le, lens; re, retina.

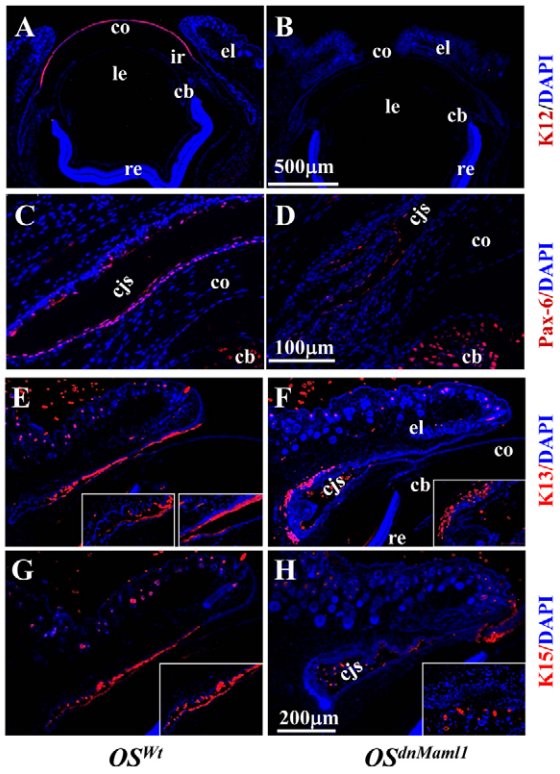


Fig. 6. Expression of dnMaml1 led to corneal epithelial cell fate change. (**A-D**) Immunofluorescent micrographs of K12 (A,B) and Pax6 (C,D) in P16 ocular surface tissues. Both K12- and Pax6-positive cells were dramatically decreased in $OS^{dnMaml1}$ (B,D) when compared with those in the OS^{Wt} littermate control (A,C). Pax6 expression level in the ciliary body was not affected. (**E-H**) Immunofluorescent micrographs of ocular surface tissues probed with anti-K13 (E,F) or anti-K15 (G,H) antibody. Both K13- and K15-positive cells were correctly detected in normal conjunctival epithelium, respectively (E,G), but dramatically decreased in $OS^{dnMaml1}$ (F,H). K13-positive signal in $OS^{dnMaml1}$ was lost from conjunctival epithelium and displaced into stroma (F); K15-positive cells were desquamated into conjunctival sac (H). Insets show higher magnifications of each panel. cb, ciliary body; cjs, conjunctival sac; co, cornea; el, eyelid; ir, iris; le, lens; re, retina.

mice (compare Fig. 7B with 7A). At P16, epidermal metaplasia was noted in that aberrant K10 expression could be detected beyond the mucocutaneous junction and extended to palpebral conjunctiva and in the central cornea of $OS^{dnMaml1}$ mice, whereas K10 expression was restricted at mucocutaneous junction of wild-type littermates (compare Fig. 7D with 7C). It is of interest to note that a pulse Dox induction from P0 to P16 resulted in persistent ocular surface pathology characterized by severe ulceration and neovascularization in the cornea, even after 180 days post-Dox induction (supplementary material Fig. S4B). Perhaps conjunctival epithelial progenitor/stem cells were depleted owing to the persistent pathogenesis resulting from the mis-expression of dnMaml1. Taken together, these results suggest that the corneal squamous metaplasia and corneal ulceration is secondary to the persistent pathology caused by the lack of goblet cells in $OS^{dnMaml1}$ mice.

dnMaml1 induced conjunctival goblet cell depletion in adult mice

Subsequently, we asked whether dnMaml1 could deplete existing goblet cells from the adult conjunctiva. Adult *K14-rtTA/TC/$Rosa^{LSL\text{-}dnMaml1}$* triple-transgenic mice were administered Dox chow at P90. We noticed that hair loss occurred 1 month after Dox induction, with obvious random alopecia on the body, including the eyelid within 3 months (Fig. 8B). PAS staining and Muc5ac immunostaining clearly showed that goblet cells lost from the conjunctiva and cellular debris scattered in the conjunctival sac of the $OS^{dnMaml1}$ mice (compare Fig. 8D with 8C, and Fig. 8F with 8E). This result indicates that canonical Notch signaling is not only required for goblet cell differentiation during eye morphogenesis at early developmental stages (embryonic and neonate), but is also indispensable for the replenishment and maintenance of ocular surface goblet cells in adult mice.

Conditional deletion of *Notch1* from ocular surface epithelia recapitulated the phenotypes of $OS^{dnMaml1}$ mice

It has previously been reported that conditional *Notch1* gene-deletion by *K14-Cre* or *K5-Cre^{ERT}* resulted in diffuse alopecia in the skin, malformation in the Meibomian gland of the eyelid, and failure of maintaining corneal epithelium-type differentiation

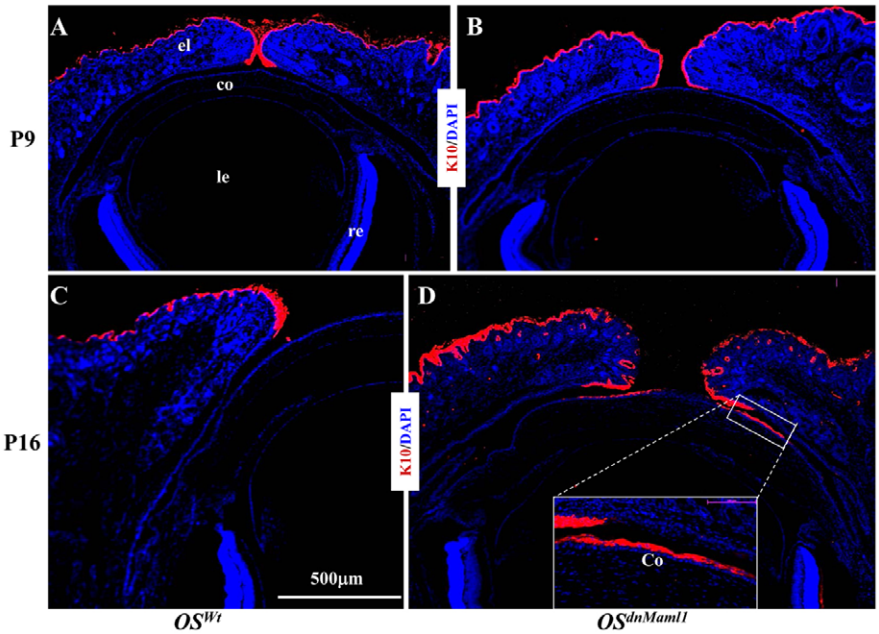


Fig. 7. Conditional expression of dnMaml1 resulted in epidermal metaplasia of corneal epithelium. (**A-D**) Immunofluorescent micrographs of K10 in ocular surface tissues at P9 (A,B) and P16 (C,D). K10-positive signal was not observed at P9 (A) but obviously appeared in $OS^{dnMaml1}$ at P16 (D). Abbreviations: co, cornea; el, eyelid; le, lens; re, retina.

following wound-healing (Vauclair et al., 2007); however, phenotypes concerning conjunctival goblet cell differentiation and maintenance were not mentioned. Given that Notch1 signaling is very active in the adult conjunctiva, revealed by the *N1IP-Cre/Rosa*$^{mTmG/Wt}$ mice at P30 (Fig. 1C), it is very likely that the conditional ablation of *Notch1* via the Cre/loxP system using *K14-rtTA* driver mice should yield phenotypes resembling those by overexpression of dnMaml1, as described above. Predictably, conditional deletion of *Notch1* in ocular surface epithelia ($OS^{N1\text{-}cko}$) using the *K14-rtTA/TC* system upon Dox treatment also led to a significant loss of conjunctival goblet cells in $OS^{N1\text{-}cko}$ animals. Interestingly, our results demonstrated that $OS^{N1\text{-}cko}$ mice had poor tear volume when compared with the wild-type littermates (Fig. 9B,C), in addition to hair loss on the skin and eyelids (Fig. 9A, mouse 3 and mouse 4). Histological examination revealed that, in the $OS^{N1\text{-}cko}$ mice, this phenotype was attributable to the reduction of goblet cells (comparing Fig. 9F with 9D, and Fig. 9G with 9E) and the degeneration of ocular glands, including lacrimal (Fig. 10) and Meibomian glands [not shown, but consistent with the report by Vauclair et al. (Vauclair et al., 2007)].

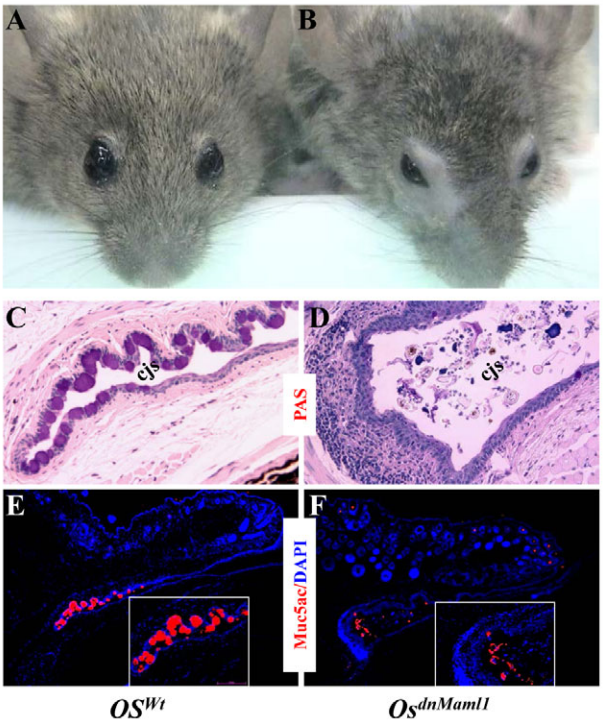


Fig. 8. Conditional expression of dnMaml1 depleted pre-existing goblet cells from conjunctiva in adult mouse. (**A-F**) *K14-rtTA/TC/Rosa*^{dnMaml1} triple-transgenic mouse (B) and *TC/Rosa*^{dnMaml1} littermate (A) fed with Dox since P90. Photos were taken at P180 and clearly showed hair loss on the ocular surface (B). Histological examination demonstrated that $OS^{dnMaml1}$ resulted in conjunctival epithelial hyperplasia, inflammation and loss of PAS-positive (compare D with C) and Muc5ac-positive (compare F with E) goblet cells. Abbreviation: cjs, conjunctival sac.

dnMaml1 downregulated expression of Krüppel-like factors and Muc5ac

It has previously been reported that the conditional knockout of Krüppel-like factors 4 or 5 (*Klf4* or *Klf5*) in the ocular surface epithelia with *Le-Cre* driver also caused a failure of goblet cell formation (Swamynathan et al., 2007; Kenchegowda et al., 2011). This result prompted us to test whether dnMaml1 could impact the expression of *Klf4* and *Klf5*. Immunofluorescence staining showed that indeed the expression levels of both Klf4 and Klf5 were significantly reduced in the conjunctival epithelium of the $OS^{dnMaml1}$ mice (Fig. 11C,D; supplementary material Fig. S5C) when compared with the OS^{Wt} littermates (Fig. 11A,B; supplementary material Fig. S5A,B). RT-qPCR analysis also indicated that *Klf4* mRNA was downregulated in $OS^{dnMaml1}$ mice (Fig. 11E). ChIP assays with NIH3T3 cells verified that RBP-Jκ could directly bind to the *Klf4* promoter *in vitro* (Fig. 11F). Luciferase assays using HEK293 cells showed that transfection of *mN1-ICD* cDNA enhanced *mKlf4* promoter activity by 2- to 4.5-fold (Fig. 11G). Furthermore, ChIP assays also indicated that Klf4 could directly bind to the Klf4-binding motif (CACCC) in the human *MUC5AC* promoter region (Fig. 11H). Transfection of *Klf4* cDNA boosted mouse *Muc5ac* promoter activity by over 1000-fold

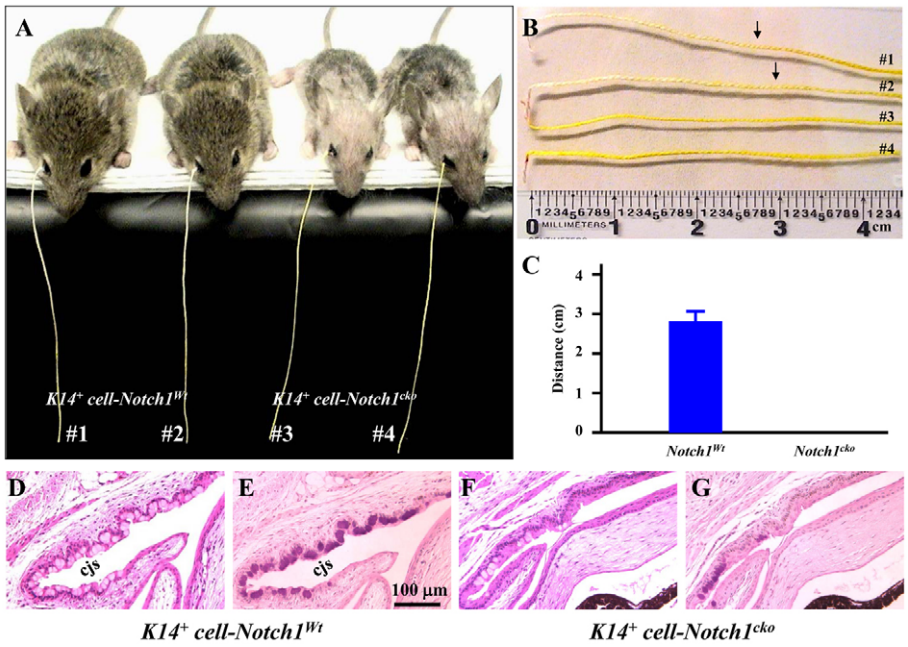


Fig. 9. Conditional knockout (cKO) of Notch1 in ocular surface recapitulated OS^{dnMaml1} phenotypes and manifested dry eye syndrome. (**A**) *K14-rtTA/TC/Notch1$^{flox/flox}$* mice (*K14^{+} cell-Notch1^{cKO}* #3, #4) and *TC/Notch1$^{flox/flox}$* littermates (*K14^{+} cell-Notch1^{wt}* #1, #2) were fed with Dox from P1 to P16 and then changed to regular chow. Photos were taken at P21 and clearly showed diffuse alopecia in *Notch1^{CKO}* mice. (**B**,**C**) Phenol red thread (PRT) test for measuring tear volume clearly showed that loss of Notch1 in ocular surface manifested no tears detected when compared with the wild-type littermates in which amount of tears turned PRT yellow into pink at 2.8 cm. Data are mean±s.e.m. (**D-G**) Histological examinations, including Hematoxylin and Eosin staining (D,F) and PAS staining (E,G) revealed that conjunctival goblet cells decreased dramatically in number in *Notch1^{cKO}* (F,G) when compared with those in *Notch1^{Wt}* mice (D,E). Abbreviation: cjs, conjunctival sac.

in HEK293 cells (Fig. 11I). Collectively, these data imply that the fine-tuning of *Klf4* by Notch activity can greatly augment *Muc5ac* gene expression, and goblet cell differentiation, during perinatal ocular surface morphogenesis.

DISCUSSION

In this study, we have investigated the phenotypes resulting from the inactivation of canonical Notch signaling by mis-expression of dnMaml1, the pan-Notch inhibitor, in ocular surface epithelia. Physiological Notch1 activation is dynamically regulated during ocular surface morphogenesis. Notch1 activation occurred in most of ocular surface epithelial cells from E15 to P1 (Fig. 1A,B). However, Notch activity remained robust in conjunctiva, but inactive in the limbus/cornea at P30 (Fig. 1C), suggesting that Notch signaling has an important role in conjunctival morphogenesis. The expression of multiple Notch family members suggests possible redundancy among different Notch ligands and receptors in the ocular surface tissues (Ma et al., 2007; Djalilian et al., 2008; Mantelli et al., 2009). Therefore, we employed a dnMaml1 overexpression strategy using triple-transgenic *K14-rtTA/TC/Rosa$^{LSL\text{-}dnMaml1}$* mice, in which excess dnMaml1 competes with endogenous Maml for binding to NICD/RBP-Jκ complexes in K14-positive stratified epithelia, including the ocular surface. This competition abrogates Notch signaling elicited from all possible Notch ligand-receptor interactions. Although it has been established that Maml1 not only binds to NICD but also interacts with various other transcription factors, namely Mef2c (Shen et al., 2006), p53 tumor suppressor (Zhao et al., 2007), β-catenin (Alves-Guerra et al., 2007) and NF-κB (Jin et al., 2010). However, Mef2c is a crucial muscle-specific transcription factor, which is not expressed in K14-positive epithelial cells. Both Maml1/p53 and Maml1/NF-κB interactions involve the N-terminal region of Maml1 (amino acids 72-301) (McElhinny et al., 2008; Jin et al., 2010), which is not present in the dnMaml1. Likewise, β-catenin binds to the C-terminal region of Maml1 (amino acids 640-1016) (Alves-Guerra et al., 2007). Predictably, dnMaml1 containing 62 amino acids (amino acids 13-74) peptide would not bind to these factors and abrogate their normal functions in K14-positive cells. Moreover, our data showed that conditional deletion of Notch1 by *K14-rt/tetO-Cre* driver partially recapitulated the phenotypes of goblet cell malformation (Fig. 9F,G). Therefore, the phenotype observed in this study is probably due to the direct abrogation of the NICD/MAML/RBP-Jκ-mediated canonical Notch pathway. However, we do not know whether the phenotypes were directly or indirectly associated with *Hey1* downregulation. Further studies are needed to elucidate the possibility.

Role of Notch signaling on cell proliferation and differentiation of ocular surface epithelia

At the cellular level, the presence of dnMaml1 abrogated Notch signaling, promoted cell proliferation (Fig. 4B,D) and led to aberrant epithelial desquamation in ocular surface (Fig. 3). A TUNEL assay did not detect any apoptotic cell in ocular surface epithelium of OS^{dnMaml1} (data not shown); however, it remained unknown whether existence of dnMaml1 might affect conjunctival epithelial cell adhesion. Notably, the presence of dnMaml1 significantly elevated ΔNp63 expression levels (Fig. 4D), but also altered K13 and K15 expression patterns in conjunctiva (Fig. 6), suggesting that OS^{dnMaml1} perhaps triggered conjunctival epithelial cells to transition to transient amplifying (TA) cell status, thus inhibiting goblet cell differentiation (Nguyen et al., 2006a; Sakamoto, et al., 2012). It is likely that the pathological manifestations of OS^{dnMaml1} mice may be initiated by changing the conjunctival epithelial identity and the loss of conjunctival goblet cells during development and in the adult. In wild-type mice, the presence of ocular surface goblet cells can be easily identified by PAS staining and immunostaining with anti-Muc5ac antibody in conjunctiva around P7-P9. These cells quickly increase in number and form clusters during the period of eyelid opening at P13-P15 (supplementary material Fig. S3). By contrast, conjunctival goblet cells were never identified in OS^{dnMaml1} mice from P9-P16 (Fig. 5). Dox induction during at P0-P16 resulted in permanent damage of ocular surface tissues. This could be, in part, explained by the persistent inflammation during the postnatal life of the experimental mice, a scenario resembling the severe dry eye syndrome in congenital disease, e.g. Sjögren syndrome (Sjögren,

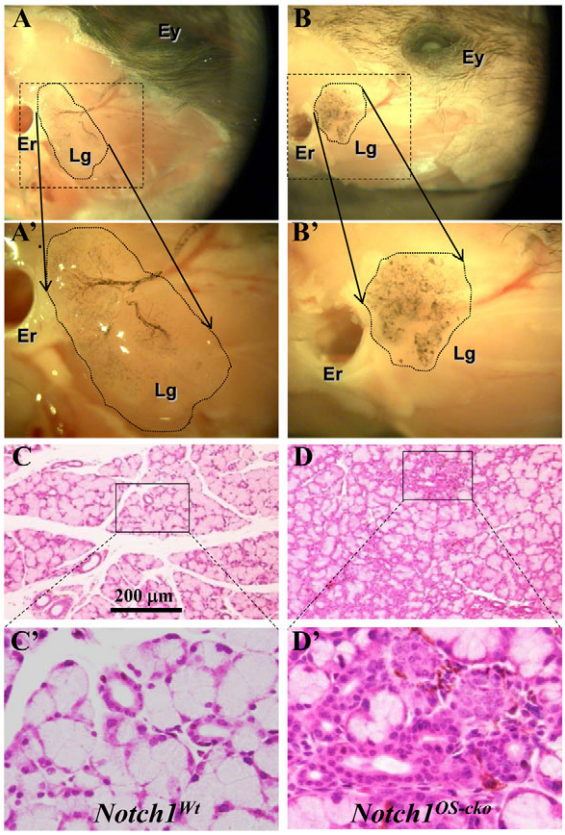


Fig. 10. Conditional knockout (cKO) of Notch1 in ocular surface caused lacrimal gland degeneration. (**A-B′**) *Notch1*^{wt} (A) and *Notch1*$^{OS\text{-}cKO}$ (B) mice fed with Dox from P1 to P16. Photos were taken at P21 and showed that the size of the lacrimal gland was significantly decreased in *Notch1*$^{OS\text{-}cKO}$ (compare B′ to A′). (**C-D′**) Hematoxylin and Eosin histological staining revealed that acinus of the lacrimal gland was infiltrated with monocytic cells and intralobular ducts were narrowed in *Notch1*$^{OS\text{-}cKO}$ (compare D with C and D′ with C′). Abbreviations: Er, ear; Ey, eye; Lg, lacrimal gland.

1933). Likewise, the overexpression of dnMaml1 in OS^{dnMaml1} mice led to complete depletion of existing goblet cells in adult conjunctiva. This result indicates that canonical Notch signaling plays a positive role in conjunctival goblet cell differentiation during and after development.

Interestingly, this finding deviates from what has been found in gastrointestinal epithelia, in that Notch acted in opposing ways at two points in intestinal goblet cell development. For example, activation of Notch is associated with promotion of cell proliferation and the suppression of intestinal goblet cell differentiation during development (Milano et al., 2004; van Es et al., 2005; Okamoto et al., 2009; Pellegrinet et al., 2011). By contrast, conditional expression of an activated Notch mutant in adult mice could turn post-mitotic intestinal epithelial cells into goblet cells (Zecchini et al., 2005). Similarly, the role of Notch signaling in the formation and maintenance of lung goblet cells also remains controversial. For example, Tsao et al. reported that ablation of canonical Notch signaling resulted in goblet cell metaplasia (Tsao et al., 2009; Tsao et al., 2011), whereas other investigators found Notch signaling promoted airway mucous metaplasia (Guseh et al., 2009; Kang et al., 2011; Rock et al., 2011). Nonetheless, perhaps the difference of cell fate changes promoted by Notch signaling might be due to the variegation of cell contexts between simple and stratified epithelia, and possibly the level of enhanced Notch signaling might have a role in the variegation of phenotypes reported.

K14 expression begins at E14 in the ocular surface epithelia (Kurpakus et al., 1994), but we did not find any significant pathological manifestations in OS^{dnMaml1} mice until P9 owing to the fact that the formation of goblet cells commences at P7 (supplementary material Fig. S4). Whereas K13 expression dramatically downregulated in the conjunctiva, K12 expression remained normal in the cornea of OS^{dnMaml1} mice (Fig. 3D). Moreover, when dnMaml1 was conditionally expressed in the corneal epithelium of Dox-induced *K12*$^{rtTA/rtTA}$*/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple-transgenic mice, no abnormality was noted during embryonic development or in the adult (Y.Z. and C.-Y.L., unpublished). This was probably due to the fact that *K12*$^{rtTA/rtTA}$/TC mice could only drive the expression of dnMaml1 in differentiated corneal epithelia, but not in limbal basal stem cells. These data suggest that Notch signaling does not have a significant enough role in the differentiation of ocular surface epithelium to be responsible for K12^{+} corneal type epithelium differentiation. Furthermore, Notch1 activation is dormant in the adult cornea (Fig. 1C), indicating that perhaps its function is dispensable in naïve cornea. However, it has been reported that Notch1 signaling is activated during corneal epithelial debridement wounding (Vauclair et al., 2007; Ma, et al., 2011); as such, it would be informative to check whether corneal epithelial cells can express GFP, which would provide evidence of Notch1 activation, in *N1IP-Cre/Rosa*$^{mTmG/Wt}$ transgenic strains during corneal epithelial abrasion wound healing. If this proves to be the case, experiments will need to be designed to examine whether dnMaml1 expression can compromise corneal epithelial wound healing in a corneal epithelial injury model. Collectively, these data suggest that conjunctival epithelia are more vulnerable than corneal epithelia in response to cellular inactivation of Notch by dnMaml1.

Ocular surface pathogenesis of OS^{dnMaml1} mice

It has been previously shown that chronic ocular surface inflammation and goblet cell disappearance usually go hand-in-hand (Ueta et al., 2005); however, their cause-and-effect relationship remains unknown. In the present animal model, our results precluded the likelihood of inflammation causing the loss of goblet cell in conjunctiva, because we did not find CD45^{+} inflammatory cell infiltration into the sub-conjunctival space of OS^{dnMaml1} until P16, but Muc5ac^{+} goblet cells had never been detected throughout the Dox-treated *K14-rtTA/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple-transgenic mice. By contrast, our data suggest that mucin deficiency due to the failure of goblet cell formation might initiate the dryness of the ocular surface and hyperosmolarity upon eyelid opening, which in turn can cause corneal irritation and inflammation, as well as compromise the corneal function in wound healing, leading to epidermal metaplasia as a secondary effect.

Molecular cascades of Notch signaling in maintenance of goblet cell functions

In this report, our results demonstrate significant downregulation of Klf4 in OS^{dnMaml1} mice (Fig. 11A-E) and NICD trans-activated *Klf4* promoter activity *in vitro* (Fig. 11G). Indeed, the mouse *Klf4* (GenBank AY071827.1) contains one potential RBP-Jκ-binding motif (GTGGGAA) (Fig. 11G) (Tun et al., 1994; Del Bianco et al., 2010); thus, Notch may directly bind and upregulate *Klf4* gene

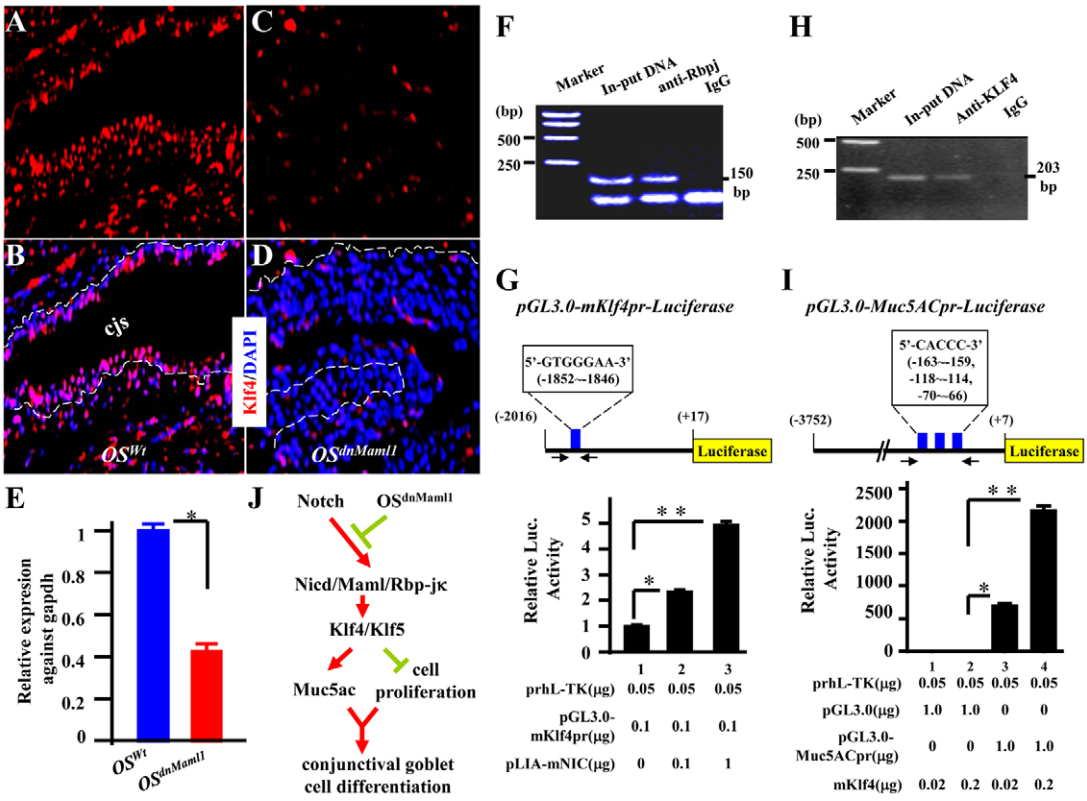


Fig. 11. OS^{dnMaml1} impaired conjunctival goblet cell formation by downregulating Klf4. (**A-D**) Immunofluorescent stainings showed that nuclear Klf4 expression levels were reduced dramatically in OS^{dnMaml1} at P9 (compare C,D with A,B). (**E**) RT-qPCR revealed that *Klf4* mRNA levels in the OS^{dnMaml1} were reduced ~60% when compared with that of the OS^{wt} littermate. (**F**,**G**) Notch activation directly upregulated the *Klf4* promoter. ChIP assays showed that anti-RBP-Jκ antibody, but not normal IgG, brought down a 150 bp DNA fragment amplified by adjacent PCR primers flanking GTGGGAA of the mouse *Klf4* promoter region (GenBank AY071827.1) in NTH3T3 cells. Dual-luciferase assays demonstrated that mouse NICD cDNA (0.1 and 1 μg) increased transactivation of mouse *Klf4* promoter activity by two- to fivefold, respectively. (**H**,**I**) ChIP assay showed that anti-Klf4 antibody precipitated a 203 bp DNA fragment amplified by adjacent PCR primers flanking CACCC of the human *MUC5AC* promoter region in NCI-H292 cells. Three Sp1-binding motif CACCC-containing sequences (Wang et al., 2004) were located in (approximately) –163 to –159 nucleotide, –118 to –114 nucleotide and –70 to –66 nucleotide regions relative to the TSS of the human Muc5ac promoter (Li et al., 1998) (GenBank AF016834). Transfection of mouse *Klf4* cDNA (0.02 and 0.2 μg) upregulated mouse *Muc5ac* promoter activity by 700 to 2200 fold in HEK293 cells. (**J**) A proposed model depicting that Notch signal activates Klf4, which in turn promotes Muc5ac synthesis, and negatively regulates cell growth and stimulates goblet cell differentiation. OS^{dnMaml1} blocks the canonical Notch pathway, downregulates *Klf4* expression, impairs Muc5ac synthesis and results in the loss of goblet cells. Data are mean±s.e.m. **P*<0.05; ***P*<0.01.

expression. Moreover, Klf4 directly binds and tremendously enhances Muc5ac promoter activity (Fig. 11H,I). Collectively, our findings suggested that the fine-tuning of Klf4 or Klf5 via Notch activation will greatly impact transcriptional activation of Muc5ac. Therefore, our results argue that the detrimental effect on the ocular surface resulting from OS^{dnMaml1} might be initiated by downregulation of *Klf4* and *Klf5*, which in turn triggers hyperplastic transformation of conjunctival epithelium and blocks *Muc5ac* transcription and goblet cell differentiation (Fig. 11J). As a consequence, ocular surface homeostasis is compromised and leads to an epidermal default pathway.

Downregulation of Muc5ac and the reduction of goblet cell density have been associated with a vitamin A-deficient ocular surface epithelium (Tei et al., 2000). Notch1 signaling has been linked to vitamin A metabolism by regulating the expression of cellular retinol binding protein 1 (Crbp1), which is required to generate a pool of intracellular retinol (Vauclair et al., 2007). These data imply that the presence of a Notch→retinol→Muc5ac signaling axis is of paramount importance for the maintenance of conjunctival goblet cells to ensure ocular surface homeostasis. Further studies in the elucidation of the link between Klf4/5 and retinol will help unravel the mechanism underlying transcriptional control of goblet cell morphogenesis. Moreover, our novel *K14-rtTA/TC/Rosa*$^{LSL\text{-}dnMaml1}$ triple transgenic mouse model may prove crucial for elucidating the pathogenesis of Notch de-regulation initiated keratoconjunctivitis sicca (dry eye) syndrome.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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