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Perturbed meibomian gland and tarsal plate morphogenesis by excess TGFα in eyelid stroma

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Abstract

Transforming growth factor alpha (TGFα) belongs to the epidermal growth factor (EGF) family and is known to play an important role during eyelid morphogenesis. In this study, we showed that ectopic expression of TGFα in the stroma of *Kera-rtTA/tet-O-TGFα* bitransgenic mice results in precocious eye opening, abnormal morphogenesis of the meibomian gland, tendon and tarsal plate malformation and epithelium hyperplasia. TGFα did not change proliferation and differentiation of meibocytes, but promoted proliferation and inhibited differentiation of the tarsal plate tenocytes. These results suggest that proper formation of the tendon and tarsal plate in the mouse eyelid is required for normal morphogenesis of the meibomian gland.

Keywords

TGFα; Meibomian gland morphogenesis; Tarsal plate; ECM; eyelid development

Introduction

The meibomian glands are one of the most important secretory glands in the eyelid. They are specified sebaceous glands, which are arranged in parallel along the tarsal plate of the upper and lower eyelids. Meibomian glands are pivotal in maintaining the health of the ocular surface by secreting meibum, which forms a lipid barrier preventing aqueous evaporation; maintaining normal tear film composition and function. Meibomian gland dysfunction (MGD) is a main cause of dry eye syndrome, leading to increased evaporation, friction and the onset of inflammation at the ocular surface (Foulks & Bron, 2003; Goto, 2003; Lemp, Crews, Bron, Foulks, & Sullivan, 2012). Therefore, the formation and maintenance of functional meibomian glands is of paramount importance for maintaining a healthy ocular

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surface. To date, the cellular and molecular mechanisms that regulate meibomian gland formation and maintenance remain unknown.

In humans, meibomian glands are first detected at 18 weeks of gestation (Byun, Kim, Park, & Kim, 2011). During mouse embryogenesis, the meibomian gland formation commences at embryonic day 18.5 (E18.5) and completes around postnatal day 15 (P15) (Nien, Massei, Lin, & Liu, 2010). Formation of the meibomian gland primordium begins at the edge of the eyelid as the ectoderm epithelium aggregates. The epithelial cells then invaginate into the eyelid mesenchyme, elongate, branch and finally differentiate into a mature meibomian gland at about 2 weeks of age concurrent with eye opening. Accompanied with the maturation of the meibomian glands, the mesenchyme around the gland develops into connective tissue, becoming the tarsal plate. Structurally, the tarsal plate is an extension of the eyelid tendon. In the upper eyelid, it consists of the Whitenall ligament and levator aponeurosis (LA), while in the lower eyelid the corresponding structures are the Lockwood ligament and caposulopalpebral fascia (CPF) (Jordan, Mawn, & Anderson, 2012). The mature tarsal plate is composed of both fibrous components like collagen I, collagen III and versican, as well as cartilaginous components including aggrecan, chrondroitin 4 sulfate and chrondroitin 6 sulfate glycosaminoglycan (Milz, Neufang, Higashiyama, Putz, & Benjamin, 2005). Therefore it provides a suitable scaffold and microenvironment for the formation of the meibomian gland. To date, little is known about the role of the tarsal plate in meibomian gland morphogenesis.

Transforming growth factor α (TGFα), a small polypeptide belonging to the epidermal growth factor (EGF) superfamily, is a mitogen for both epithelial and mesenchymal cells. TGFα is critical during eyelid development. Targeted gene disruption of the TGFα or EGF receptor results in a failure of prenatal eyelid fusion, which is associated with disorganization of the orbicularis muscle and absence of the meibomian glands (Noreen C Luetteke et al., 1993; Mann et al., 1993; Miettinen et al., 1995). On the other hand, overexpression of TGFα in the epidermis of transgenic mice results in precocious postnatal eye opening (Dominey et al., 1993; Vassar & Fuchs, 1991) and a thicker epidermis. Studies have shown that the TGFα/EGFR signaling pathway is responsible for epithelial cell proliferation and migration at the eyelid tip, yet there is no direct evidence showing that TGFα is associated with eyelid mesenchymal cell development. Hayashi et al. demonstrated that excess biglycan sequestered TGFα and abolished TGFα-induced periocular mesenchymal cell migration in *Kera-Bgn* transgenic mice, suggesting that TGFα may be involved in periocular mesenchyme development (Hayashi et al., 2005). In the present study, a doxycycline (dox) inducible bi-transgenic mouse model, *Kera-rtTA/tetO-TGFα* (*KR/TG*), was used to determine whether ectopic expression of TGFα by periocular mesenchymal cells would affect eyelid development. Our data show that excess TGFα secreted by eyelid mesenchymal cells perturbs eyelid morphogenesis accompanied by precocious eye opening, meibomian gland atrophy, cyst formation in the tarsal plate and eyelid tendon and epithelium thickening. We show that TGFα did not affect the proliferation and differentiation of meibocytes, but promoted proliferation and inhibited differentiation of the tarsal plate tenocytes. These results suggest that proper formation of the tendon and tarsal plate in the mouse eyelid is required for normal morphogenesis of the meibomian gland.

Materials and methods

Generation and Identification of Bi-transgenic *Kera-rtTA/tetO-TGFα* Mice

Bi-transgenic *Kera-rtTA/tetO-TGFα* (*KR/TG*) mice were generated *via* mating *Kera-rtTA* (*KR*) transgenic mice (Zhang, Kao, Pelosi, Schlessinger, & Liu, 2011) and *tetO-TGFα (TG)* transgenic mice (Hardie et al., 2004). KR is a driver line expressing reverse tetracycline-controlled transactivator (rtTA) by the keratocan promoter in periocular mesenchymal cells (Liu, Arar, Kao, & Kao, 2000). When induced with doxycycline, rtTA, encoded by the *Kera-rtTA* transgene, will bind to the TRE (tetracycline response element) of the tetO operon of the *tetO-TGFα* transgene and initiate synthesis of TGF*α* in periocular mesenchyme. Newborn pups of heterozygous bi-transgenic *KR/TG* mice containing one single allele of each individual *KR* and *TG* transgenes as well as single-transgenic littermate controls were subjected to dox induction by feeding nursing mothers with 1 g/kg dox chow (Custom Animal Diets, Bangor PA) from postnatal day 0 (P0, at birth) or embryonic day 0 (E0) through various time points.

The eyes of experimental mice induced from P0 were collected at various time points (e.g., P0, P5, P8, P11, P15) and subjected to histological and immunofluorescence staining. Eyelids of 13 bi-transgenic *KR/TG* mice induced from P0 to P15 were collected to analyze the rate of penetrance. Another 7 bi-transgenic mice and 6 single transgenic control mice induced from E0 were divided into two groups: Group1 (3 bi-transgenic and 3 control mice) was subjected to continuous induction; Group 2 (4 bi-transgenic mice and 3 control mice) had induction terminated at P21 and fed with normal chow. Mice from both groups were collected at P54 and subjected to histological and immunofluorescence staining. All mice were housed at the Animal Facility of the University of Cincinnati College of Medicine. Experimental procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of experimental animals in vision and ophthalmology research and were approved by the Institutional Animal Care and Use Committee, University of Cincinnati.

Transgenic mice were identified by polymerase chain reaction (PCR) of tail DNA using the following primers: Forward KR (primer 1): 5′-TCAGCCATCGCTATGACTCAGTTC-3′, Reverse KR (primer 2): 5′-TTGTTCTTCACGTGCCAGTACAGG-3′ for detecting the *KR* transgene; Cytomegalovirus (CMV) minimum promoter forward primer, 5′-GTC AGA TCG CCT GGA GAC GCC-3′, reverse primer in hTGFα, 5′-CGT GGT CCG CTG ATT TCT TCT CTA-3′ for detecting the *tetO-TGFα* transgene.

Histological analysis

Specimens were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C, followed by paraffin or cryo embedding. De-paraffinized sections (5 μm) were stained with Masson's Trichrome and hematoxylin/eosin (H&E) and examined with a Nikon ECLIPSE E800 microsocpe.

Immunohistofluorescence (IF) staining

Paraffin sections (5 μm) were deparaffinized, rehydrated and subjected to antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). Sections were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature, then incubated overnight at 4°C with the primary antibodies diluted in 1% BSA. The following primary antibodies were used in the study: rabbit anti-PPARγ monoclonal antibody (2435; Cell Signaling), rabbit anti-α Smooth Muscle Actin antibody (ab5694; Abcam), rabbit anti-N-cadherin antibody (04-1126; Millipore), mouse anti-myosin antibody (ms1236; Thermo Fisher Scientific), rabbit anti-collagen I antibody (ab34710; Abcam), rabbit anti-collagen III antibody (ab7778; Abcam), rabbit anti–proliferating cell nuclear antigen (PCNA) antibody (ab2426; Abcam), rabbit anti-EGFR antibody (06-847; Millipore). After three washes in PBS, slides were incubated at room temperature for 1 h with Alexa 647-conjugated secondary antibodies (Life Technologies). Nuclei were counterstained with 1 ng/ml 4′, 6-diamidino-2-phenylindole (DAPI) and mounted with Mowiol (475904; Calbiochem). Sections were examined and photographed using a Zeiss microscope, Axio Observer Z1, equipped with an apotome and camera (Axiocam; Carl Zeiss GmbH, Oberkochen, Germany).

Western blot analysis

Western blot was performed to verify the expression of TGFα in experimental animals. The transgene usually starts to express 24-48 hours after induction. In order to have the transgene fully expressed and get enough tissue, mice at P8 were euthanized and the eyelids were immediately dissected and placed in 400 μl PBS containing 3% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) and protease inhibitors. The eyelids were then homogenized and centrifuged. Protein concentration was determined by BCA Protein Assay Kit according to the manufacturer's instruction (Thermo Scientific™ Micro BCA Protein Assay Kit, cat# 23235). An equal amount of protein (30 μg) was loaded into each well of a 4%–12% Bis-Tris polyacrylamide gel with MES SDS running buffer for use with small- to medium-size proteins (Invitrogen). Western blot was carried out by transferring proteins onto a PVDF membrane (Millipore) followed by immune staining using an mouse anti-TGFα antibody (MS-670-P1; Fisher Scientific) and visualization with goat anti-rabbit IgG fluorescent conjugate; normalization was done with rabbit anti-β-actin antibody (ab8227, Abcam).

Cell Lineage Tracing

Rosa26mTmG (Gt(ROSA)26Sor$^{tm4(ACTB-td(Tomato,-EGF)Luo)}$ reporter (Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007) and Wnt1Cre (129S4-Tg(Wnt1-cre)1Sor/J) (Danielian & McMahon, 1996) mice were obtained from Jackson Lab (Bar Harbor, Maine). The *KR/TG* mouse strain was crossed with the *Wnt1Cre/Rosa26*^{mTmG} *(WC/mTG)* reporter mice to get quadruple *KR/TG/WC/mTG* transgenic mice. The *KR/TG/WC/mTG* mice and their littermate controls *KR/WC/mTG* were fed with dox chow from P0 to P15.

Cryosections (10 μm thick) were prepared from quadruple *KR/TG/WC/mTG* transgenic mice and their triple *KR/WC/mTG* littermates. Sections were washed with PBS and

incubated with 1 ng/ml DAPI for 1 h. Washed 3 times with PBS then mounted with Mowiol. Sections were examined with a ZEISS Axio Observer Z1 microscope as described above.

Results

1. Postnatal overexpression of TGFα in the eyelid stroma

To conditionally overexpress TGFα in eyelid stromal cells, keratocan promoter *rtTA* (*KR*) mice and *tetO-TGFα (TG)* transgenic mice were used to create bi-transgenic *KR/TG* mice (transgenic mice) and their single-transgenic littermates *KR* and *TG* (controls) (Fig. 1A). Experimental mice were induced with dox chow from P0 to P15 as described in the Methods. Eyelids at the age of P8 were used to determine the expression level of TGFα by western blot. As shown in Fig. 1B, bitransgenic mice have a higher level of soluble TGFα (6 KD) in the eyelid. Control littermates (KR single transgenic mice) have no or little TGFα beyond the sensitivity of the test.

2. Eyelid morphogenesis is perturbed in *KR/TG* mice

2a. Precocious eye opening in *KR/TG* mice—Control eyes opened between P12 and P14 while mutant mice exhibited precocious eye opening at approximately P8-P10. Stereomicroscopic images (Fig. 2A) and HE staining (Fig. 2C) at P11 showed closed eyes with no eyelid anomalies in the control. In contrast, mutant mice at the same age exhibited open eyes and swollen eyelids, with more significant swelling in the lower eyelid than in the upper (Fig. 2B, D).

2b. Meibomian gland malformation in *KR/TG* mice—To further explore the eyelid anomaly seen in the mutant mice, meibomian gland morphology was examined. Stereomicroscopic images of control mice at P15 showed fully developed meibomian glands (Fig. 3A, MG indicated by red arrows). Morphological analysis revealed clusters of acini branching from the central duct (Fig. 3D, 3G), with the duct orifice being located anterior to the mucocutaneous junction (MCJ). In contrast, mutant mice exhibited meibomian gland anomalies with a variation of severity among individual pups. Among 13 bi-transgenic mice, 54% (7/13) displayed severely affected meibomian glands while the other 46% (6/13) exhibited a mild phenotype. Anomalies of the meibomian gland in the lower lid were more prominent than the upper lid (Fig. 3B, 3C). Therefore, all further analyses were focused on the lower lid. Severely affected meibomian glands from a mutant mouse, *KR/TG* (a), showed partial loss of acini in the lower lid (Fig. 3B, E and F). In sporadic regions there were severely affected glands exhibiting disorganized acini with a large number of mesenchymal cells accumulated around the meibomian gland (star in Fig. 3H). Additionally, the gland orifices were mislocated to the skin epidermis distant from the MCJ (Fig. 3E). Mildly affected meibomian glands, KR/TG (b), displayed only a subtle difference to that of the control (Fig. 3C, F and I) However, histological examination showed that there was still a marked increase of mesenchymal cells accumulated around the gland (star in Fig. 3F, I).

2b. Eyelid tendon and tarsal plate malformation in postnatal induced *KR/TG* mice—As mentioned above, a large number of cells accumulated around the meibomian gland of the mutant mice, which differed from the control glands that were surrounded by

the tarsal plate. The tarsal plate consists of fibrous connective tissue extending from the eyelid tendon, which is rich in glycoproteins (such as collagen). In order to analyze this phenotype in more detail, we performed Masson's trichrome staining to distinguish cells from the surrounding connective tissue. The scattered red (cytoplasm cells) and continuous blue coloration (collagen) of Masson's trichrome staining in the control mice showed a small number of fibroblasts and abundant extracellular matrix in the eyelid tendon and tarsal plate (Fig. 4A). In contrast, the tendon and tarsal plate of mutant mice had a significant increase in the number of cells but a prominent decrease in the amount of collagen (Fig. 4B). There were even more cells adjacent to the tarsal plate where cysts formed (* in Fig. 4Bb). Higher magnification revealed that the normal elongated, spindle-shaped tendinous cells in the control mice (Fig. 4Aa, Ab) were replaced by compact round cells in the mutant eyelid (Fig. 4Ba, Bb).

2c. Other phenotypes in *KR/TG* eyelid—In addition to the meibomian gland and tarsal plate malformation, mutant eyelids exhibited additional anomalies including hyperplasia. TGFα is a known mitogen of epithelial cells leading to hyper-proliferation and consistent with this notion the palpebral epithelium including epidermal, conjunctival and mucocutaneous epithelium thickened upon overexpression of TGFα (Fig. 5A, B, C, D). Immunofluorescence staining showed loss of Krt4 (differentiation marker of mucosal epithelium) expression at the MCJ (Fig. 5G, H), while Krt10 (differentiation marker of epidermal epithelium) expression extended over the MCJ onto the conjunctiva in the mutant eyelids (Supplementary Fig. 1 I, J). PAS staining showed an increase in goblet cells at the conjunctival fornix following 15 days of TGFα overexpression (Fig. 5E, F).

The expression of α-SMA (Sup Fig. 1 A) and skeletal-myosin (Sup Fig. 1 B), specific markers of tarsal muscle (TM) and orbicularis oculi (OO) respectively exhibited no difference between mutant and control mice except for some interruption of α-SMA expression at the distal part of the TM. In the lower eyelid the CPF runs next to and parallel with the TM; therefore, the break of the tarsal muscle is probably due to the presence of cysts derived from hyper-proliferative mesenchymal cells seen in Sup Fig. 1B.

3. The cell origin of the cyst in the lower eyelid

It is known that the meibomian gland is derived from ectoderm while the tarsal plate and eyelid tendon is derived from mesoderm and neural crest mesenchyme (Kao, Liu, & Zhang, 2013). To determine the cellular origin of the cysts present in the mutant mice, a neural crest reporter mouse line, *Wnt1Cre/Rosa26^{mTmG} (WC/mTG)*, was utilized. In *WC/mTG* mice neural crest derived cells express membrane bound GFP (mG) while non-neural crest derived cells express membrane bound tomato red fluorescent protein (mT). Bi-transgenic *KR/TG* mice were bred with *WC/mTG* to get quadruple transgenic *KR/TG/WC/mTG* and triple transgenic *KR/WC/mTG* mice. These mice were induced from P0 to P15. Fluorescent images of the triple transgenic *KR/WC/mTG* (Fig. 6A–G) revealed that in the lower eyelid, most of the mesenchymal cells of the tendon (B–D) and tarsal plate (E–G) were mG positive indicating the main origin of these tissues were neural crest. Interestingly, in the upper eyelid (Sup Fig. 2) only a small number of the tendon cells were derived from neural crest. These

data show that different cell lineages contribute to the formation of the lower and upper eyelid tendons.

In *KR/TG/WC/mTG* mice, both the tendon (arrow head in Fig. 6B′–D′) and the tarsal plate (Fig. 6E′–G′) were robustly thickened with a prominent increase in mG expressing cells; indicating that overexpression of TGFα stimulated proliferation of neural crest derived cells. The presence of green cells in the cyst confirmed that the cyst had the same origin as that of the tendon and tarsal plate. Negative staining of the epidermis markers pan-CK and E-cadherin and positive staining of fibronectin, a fibroblast cell marker, (Sup Fig. 3) lend additional support to the notion of the cyst being comprised of mesenchymal cells rather than epithelial cells. Interestingly, the tarsal muscle, which is also derived from cells of neural crest origin, is barely affected (arrow in Fig. 6B–D, B′–D′).

4. Meibomian gland and tendon malformation process in *KR/TG* mice

It was previously observed that formation of the meibomian glands initiates at E18.5, but most of the morphogenetic events, e.g., elongation and branching of the glands and differentiation of acinus cells take place after birth and complete at the time of eye opening (Nien et al., 2010). To examine how excess TGFα disrupts meibomian gland morphogenesis and tendon development, mice were induced at birth, P0, and collected at P0, P5, P8, P11, and P15 for histological analysis.

Consistent with Nien et al., (2010), Masson's trichrome staining revealed the eyelid as a thin fold of fused skin at P0 (Fig. 7A). At this stage, the tarsal muscle primordia has reached the anterior part of the eyelid and the tendon just underlying the tarsal muscle was not well developed as shown by the mixed blue and red colors of Masson's trichrome staining (Fig. 7Aa). Also at P0, the epidermal epithelium placode invagination into the developing mesenchyme can be readily recognized (Fig. 7Ab). The premature tarsal plate, orbicularis oculi and a few hair follicles, were in place at the time of birth. At P5 (Fig. 7B), the tendon runs parallel to the tarsal muscle (Fig. 7Ba) and attaches to the tarsal plate primordium (Fig. 7Bb). The primordium of the tarsal plate forms by condensation of mesenchymal cells. Accompanied with a lengthened tarsal plate, the meibomian glands elongated at P5 (Fig. 7Bc) and began to branch into the tarsal plate at P8 (Fig 7D). By P11 (Fig. 7F), clusters of meibomian gland acini have formed (Fig. 7Fb, Fc) and by P15 (Fig. 4A), appendages of the eyelid including hair follicles, meibomian gland, Müller's muscle and tendon have matured.

Development of the eyelid of mutant mice appears normal during the early stages of induction. The caposulopalpebral fascia (CPF) anomaly was first detected at P5 (Fig. 7C). Abnormal accumulation of mesenchymal cells was seen in the stroma at the eyelid tip. Meibomian gland invagination and elongation was hampered as evidenced by a relatively shorter gland (Fig. 7C compared to Fig. 7B, Fig. 7E compared to Fig. 7D). The tendon fibers were slightly thickened at P5 and the epidermal and conjunctival eyelid epithelia had signs of hyperplasia. By P8, a clear distinction could be seen between mutant and control eyelids (Fig. 7E). Large numbers of cells were present at the tendon region and eyelid tip (Fig. 7Ea, Eb). In severely affected eyes (Fig. 7E), cysts formed, and the meibomian gland orifice was mislocated (Fig. 7Ec). At P11 (Fig. 7G), there was a further accumulation of mesenchymal

cells at the eyelid tip as well as an increase in tendon thickening and an increase in mesenchymal cells in the eyelid stroma.

5. Proliferation, apoptosis and differentiation of meibomian gland epithelial cells

As mentioned above, morphogenesis of the meibomian gland, tendon and tarsal plate was disrupted in mutant mice. To determine if perturbed cell homeostasis may account for the observed anomaly, proliferation, apoptosis and differentiation was examined at P15. In control mice, proliferative cell nuclear antigen (PCNA), a marker of cell proliferation, was expressed in the basal cell layer of the meibomian gland (Fig. 8Aa) and scattered throughout the eyelid tendon (Fig. 8Ab) and tarsal plate (Fig. 8Ac). The pattern of PCNA expression in the mutant meibomian gland was consistent to that of the control (Fig. 8Ba); in contrast, an increase in PCNA positive cells was found in the mutant tendon (Fig. 8Bb) and tarsal plate (Fig. 8Bc). TUNEL analysis showed few acinar cells undergoing apoptosis in the meibomian glands of both control (Fig. 9Aa) and mutant mice (Fig. 9Ba). In contrast to the meibomian gland, numerous apoptotic cells were found in the mutant eyelid tendon and tarsal plate (Fig. 9Bb, Bc) compared to the control (Fig. 9Ab, Ac).

To determine if meibomian gland differentiation was affected by excess TGFα, we examined the expression of peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ is a nuclear receptor important for the regulation of fatty acid synthesis and metabolism in adipocytes and is a marker of meibomian gland differentiation (Nien et al., 2010). PPARγ was expressed in control meibomian gland acini at P15 (Fig. 10A). Although mutant mice have a defect in meibomian gland morphogenesis, the glands that do form also express PPARγ in the acini (Fig. 10B).

Differentiation of the tendon and tarsal plate were also assessed at P15. Extracellular collagen I (Fig. 10C) and collagen III (Fig. 10E) were rich in the eyelid tendon and tarsal plate of control mice; however, they were dramatically down regulated in the eyelid stroma of mutant mice (Fig. 10D, E).

Taken together, these observations suggest that excess TGFα had little effect on proliferation, apoptosis and differentiation of the meibocytes, but significantly promoted proliferation and inhibited differentiation of the tendon and tarsal plate tenocytes.

6. Mechanism of tenocyte hyperplasia by excess TGFα

TGFα is an EGFR ligand; therefore, we explored this signaling pathway. At P15, expression of EGFR (compared Fig. 11B to Fig. 11A) was dramatically enhanced by TGFα stimulation in the mutant tendon, an observation similar to what has been reported by Singh and Coffey (Singh & Coffey, 2014). While there was little EGFR expression, if any in control tendons. It is known that there is a bidirectional, positive feedback loop between the Wnt/β-catenin and EGFR signaling pathways (Georgopoulos, Kirkwood, & Southgate, 2014). Consistent with this notion, expression of β-catenin increased and nuclear translocation was seen in the periphery of the tendon (Fig. 11D).

We also found N-cadherin, a mesenchymal progenitor cell marker (Ishimine et al., 2013), strongly up-regulated by the mutant tenocytes (Fig. 11F), indicating that these cells fail to

differentiate and assume a tendon cell phenotype, but rather maintain the feature of mesenchymal progenitor cells. Seven bi-transgenic mice and six single transgenic control mice induced from E0 were divided into two groups. Mice of Group 1 (3 bi-transgenic and 3 control mice) were subjected to continuous induction while mice in Group 2 (4 bi-transgenic mice and 3 control mice) were induced until P21 (at which time induction was stopped) and chased until P54. Masson's trichrome staining of transgenic mice from Group 1 revealed the presence of cysts in the tendon and tarsal plate (Sup Fig. 4B), while no cysts were seen in mice from Group 2. In these mice, the tendon and tarsal plate formed though they were a little thicker than the control mice (Sup Fig. 4C) indicating that the cysts recovered to assume normal histology (Sup Fig. 4D). Consistent with this, N-cadherin was no longer detected in the tendon of Group 2 mice (Sup Fig. 5D), while highly expressed in the cysts of Group 1 mice (Sup Fig. 5B), suggesting an association of TGFα with sustained N-cadherin expression, resembling a tendon progenitor cell phenotype.

Discussion

In the present study, we found that overexpression of TGFα leads to precocious eye opening, epithelium hyperplasia, abnormal meibomian gland morphogenesis and malformation of the eyelid tendon and tarsal plate. Our results show that excess TGFα does not affect the proliferation and differentiation of meibocytes, but disturbs overall meibomian gland morphogenesis by promoting tenocyte proliferation and inhibiting their differentiation. Taken together these findings suggest that morphogenesis of the meibomian gland depends on the formation of a normal tarsal plate and its surrounding mesenchyme.

Mouse eyelid morphogenesis initiates at E13.5 when the eyelid protrudes from the periphery of the cornea. After fusion at E17.5, the eyelid reopens between P12 and P14 (Findlater, McDougall, & Kaufman, 1993). Studies have shown that the protein level of TGFα is responsible for eyelid morphogenesis. Either excess or deficient expression of TGFα leads to precocious eye opening in mice. Overexpression of TGFα accelerates the eyelid reopening by inducing hyper-proliferation and maturation of the eyelids while TGFα deficiency leads to underdeveloped eyelids and failure of eyelid fusion during development. Moreover, malformation of the tendon and tarsal plate may affect the reopening process of the mouse eyelid. In our study, 54% of mutant mice exhibited a severe lower eyelid phenotype while 46% of them showed a mild phenotype. Phenotypic variability is frequently observed in spontaneous human gene mutation and artificial transgenic animal models. How this phenotypic variability arises is still not fully understood, but genetic background and variable expression levels are the most likely explanation.

In mammals, tissue genesis and organogenesis such as hair, lung, mammary gland, sweat gland and lacrimal gland require interactions between the mesenchyme and epithelium (Howard & Lu, 2014; Jussila & Thesleff, 2012; Parmar & Cunha, 2004; Shannon & Hyatt, 2004). Before the first morphological indication of gland development, the mesenchyme supplies the inductive signal for the formation of the epithelium placode. Following epithelium thickening at the gland-forming region, the underlying mesenchyme condenses inducing gland primordial epithelium invagination, differentiation and branching at later developmental stages. In addition to being a physical support for tissue morphogenesis,

mesenchyme regulates tissue patterning via dynamic synthesis and remodeling of the ECM and unique signaling transduction cascades. Inappropriate extracellular matrix could impede tissue morphogenesis and threaten the homeostasis of developed tissue, eventually inducing a disease process.

During eyelid morphogenesis, two major cellular events occur: 1) Migration and differentiation of periocular mesenchymal cells from both neural crest and mesodermal origin lead to the formation of the eyelid stroma, extrinsic muscles and the tarsal plate of the eyelids; 2) Differentiation of surface ectoderm gives rise to epidermal epithelia as well as glandular epithelia (Ohuchi, 2012). Therefore, we infer that meibomian gland morphogenesis is similar to other gland development as mentioned above, needing mesenchymal-epithelial interaction. Our data suggest that tarsal plate malformation may account for the improper meibomian gland morphogenesis seen in the mutant mice; highlighting the importance of mesenchymal-epithelial interactions during meibomian gland development. Hyper-proliferation and improper differentiation of mesenchymal precursor cells may lead to malformation of the tarsal plate in addition to occupying the space essential for meibomian gland invagination and branching. The misplacement of the meibomian gland and its orifice in severely affected eyelids supports this hypothesis. Furthermore, excess TGFα in the eyelid stroma disturbs normal extracellular matrix formation including collagen I and collagen III, which may impede the proper crosstalk between the meibomian gland epithelial cells and tarsal plate mesenchymal cells, affecting development and function of the meibomian glands. Further studies are needed to elucidate the underlying mechanism(s) accounting for the meibomian gland anomaly resulting from excess TGFα.

Utilizing the neural crest reporter line, *WC/mTG*, we traced the cell origin of the tendon and tarsal plate for the first time. We found that the tendon and tarsal plate of the lower eyelid are derived mainly from neural crest cells, while in the upper eyelid most cells originate from mesoderm origin. A more obvious phenotype in the lower eyelid than the upper eyelid in the presence of excess TGFα suggests that neural crest derived mesenchymal cells are more sensitive to TGFα stimulation than mesenchymal cells from other origins, e.g. mesoderm. During embryogenesis keratocan-expressing periocular mesenchymal cells are progeny of Wnt1-positive cells; therefore, only green neural crest derived cells may express TGFα driven by the keratocan promoter. Hence the red, non-neural crest derived tissue, such as the orbicularis oculi, was not significantly affected by excess TGFα stimulation because of the low TGFα concentration. Interestingly, both the tarsal muscle and CPF (capsulopalpebral fascia) of the lower eyelid are mainly neural crest derived, but excess TGFα only affected the development of the CPF and not the tarsal muscle. This is likely due to the fact that EGFR expression is low in the tarsal muscle but extremely high in the CPF of mutant mice; implicating that TGFα activates EGFR signaling in the tenocytes but not in the tarsal muscle cells. These data suggest that excess TGFα plays an important role in determining the cell fate of neural crest derived cells. These data offer a possible explanation for the more severe abnormality observed in the lower eyelid than in the upper.

Our observation of thickened ocular surface epithelia in the mutant mice is consistent with the role of TGFα as a mitogen of epithelial cells (Huang, Sauma, Yan, & Friedman, 1995;

Luetteke & Lee, 1990; Sauma, Huang, Winawer, & Friedman, 1995). To date, little is known regarding the role of TGFα in tendon morphogenesis. Reneker et al., (1995) found that TGFα could act as a chemoattractant to regulate periocular mesenchymal cell migration in developing mouse eyes using *αA-crystallin/TGFα* transgenic mice. Several lines of evidence show that EGFR signaling plays a role in tendon cell proliferation and differentiation (Fong, Backman, Andersson, Scott, & Danielson, 2013; Martin-Bermudo, 2000). According to our data, TGFα promoted proliferation and inhibited differentiation of eyelid tendon cells. This is supported by the altered expression levels of PCNA as well as collagen I and III. Additionally we found that excess TGFα leads to cyst formation in the tendon and tarsal plate, but can be reversed after withdrawal of dox. Activated EGFR signaling and increased expression of β-catenin with some nuclear translocation may account for the increased proliferation observed in the tendon cells. Several factors like scleraxis (Scx) and tenomodulin (TNMD) have also been shown to regulate proliferation and differentiation of tenocytes (Docheva, Hunziker, Fässler, & Brandau, 2005; Shukunami, Takimoto, Oro, & Hiraki, 2006). Further studies are needed to elucidate the cellular and molecular mechanism of tendon morphogenesis in our *KR/TG* mice.

In summary, we have demonstrated for the first time that TGFα serves as a mitogen for eyelid tenocytes. Excess TGFα perturbs eyelid tendon and tarsal plate formation by promoting proliferation and inhibiting differentiation of tenocytes leading to meibomian gland malformation. Thus, it is intriguing to suggest that the tarsal plate is an important microenvironment and regulatory factor for the formation of the meibomian gland.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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highlights

- *Kera-rtTA/tet-O-TGFα* (*KR/TG*) mice were generated and induced to overexpress TGFα in eyelid stroma postnatally
- Eyelid morphogenesis is perturbed in *KR/TG* mice
- TGFα change proliferation apoptosis and differentiation of the tendon and tarsal plate tenocytes but not meibomian gland epithelial cells
- Preliminary mechanism of tenocytes hyperplasia by excess TGFα

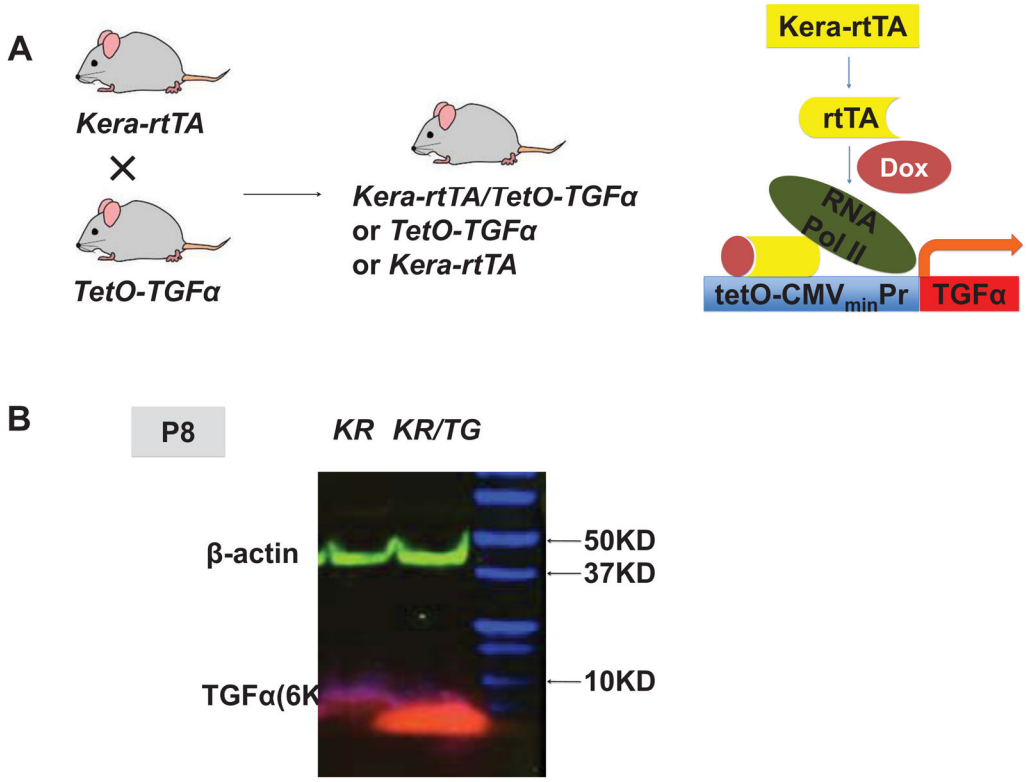

Fig. 1.

Postnatal overexpression of TGFα in eyelid stroma. The *KR/TG* bi-transgenic mouse and its littermate control were generated via mating *Kera-rtTA*, keratocan promoter driven transgenic mouse line with *tetO-TGFα* transgenic mouse line. Newborn pups of heterozygous bi-transgenic *KR/TG* mice and single-transgenic littermates were subjected to dox induction by feeding nursing mothers with 1 g/kg dox chow from postnatal day 0 (P0, at birth) through different time points. (A) Schematic showing the generation of *KR/TG* bi-transgenic mice and overexpression of TGFα after induction. (B) Western blot shows overexpression of TGFα protein in the P8 *KR/ TGFα* eyelids compared to control littermates. Dox, doxycycline.

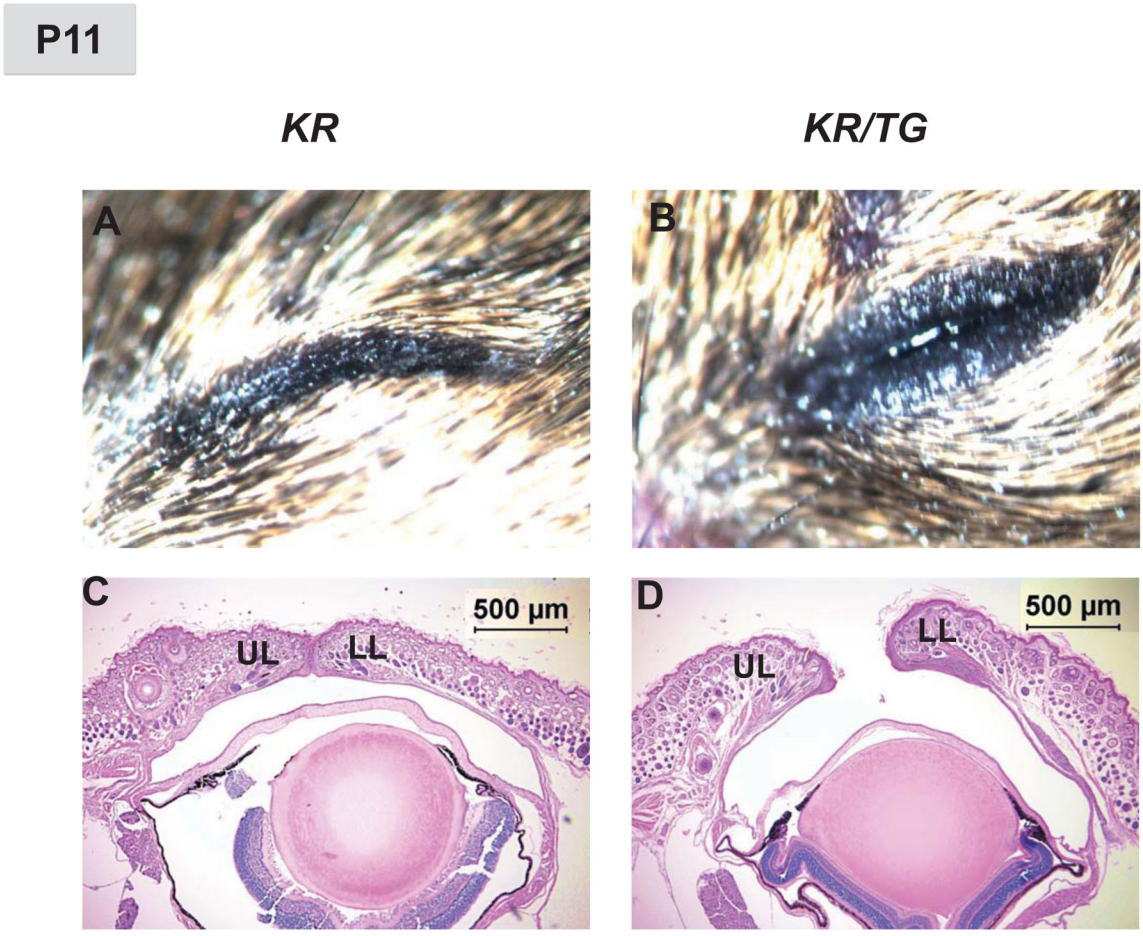

Fig. 2. Precocious eye opening of *KR/TG* mice

Eyelid morphogenesis of *KR/TG* bi-transgenic mice and single-transgenic littermates induced by dox was examined with a stereomicroscope. Eyes at different time points were fixed overnight in 4% paraformaldehyde (PFA) at 4°C, followed by paraffin embedding. Hematoxylin and eosin staining (HE) were applied to paraffin sections to show the histology of the eyelid. Stereomicroscopic images of neonates at P11, (A) eyes of control mice were closed and no anomalies were observed in the eyelid. (B) Open eyes and swollen eyelids were observed in mutant mice. HE staining (C, D) confirmed the eye open phenotype of *KR/TG* mice. The lower eyelids have a more swollen phenotype than the upper eyelids. UL, upper eyelid; LL, lower eyelid.

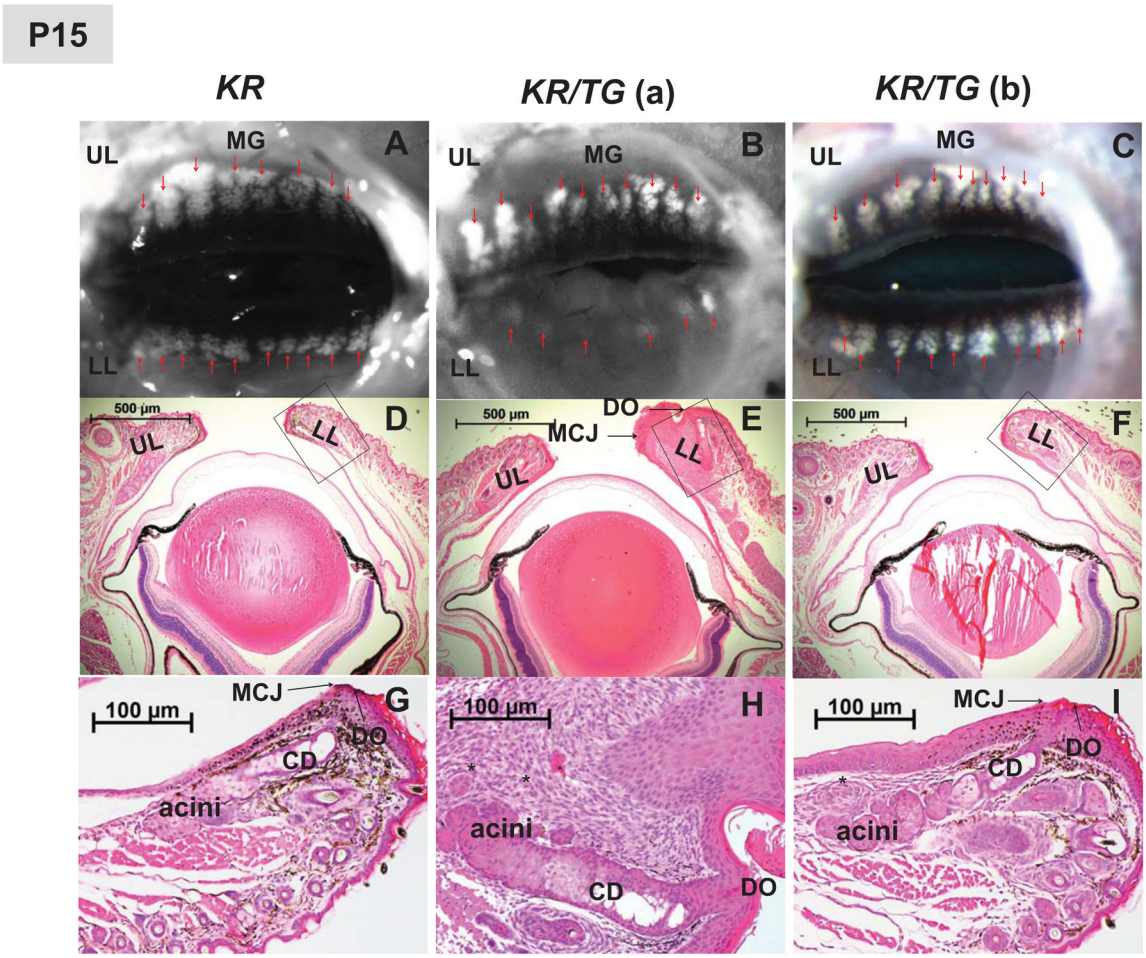

Fig. 3. Meibomian gland malformation in *KR/TG* mice

Eyelids of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were dissected and fixed in PFA at 4°C overnight. Gross images of meibomian gland morphogenesis were obtained with a stereomicroscope by directly turning over the eyelid to expose the conjunctiva. HE staining of paraffin sections of P15 mice revealed histological structures. Stereomicroscopic images (A-C) and HE staining (D-I) of P15 mice showed meibomian gland malformation with different severities at P15. Control meibomian glands (A, red arrows) showing homogeneous meibomian glands embedded in parallel along the tarsal plate; severe bitransgenic mice with Meibomian gland degenerated (B, red arrows); mild bitransgenic mice (C). (D, G) Each gland is composed of clusters of acini along the central duct; the duct orifice is anterior to the mucocutaneous junction (MCJ). A representative image of a severely affected eyelid (B) partial loss of meibomian glands in the lower lid. In sporadic regions glands formed, (E, H) exhibiting disorganized acini and pronounced dilated ducts, misplacement of gland orifices and a large number of accumulated mesenchymal cells (* in H). A representative image of a mildly affected eyelid displayed little difference to their control littermates upon gross observation (C). However, there were still a marked number of mesenchymal cells accumulated around the gland (* in

I). UL, upper eyelid; LL, lower eyelid; MCJ, mucocutaneous junction; DO, duct orifice; CD, central duct; MG, meibomian gland.

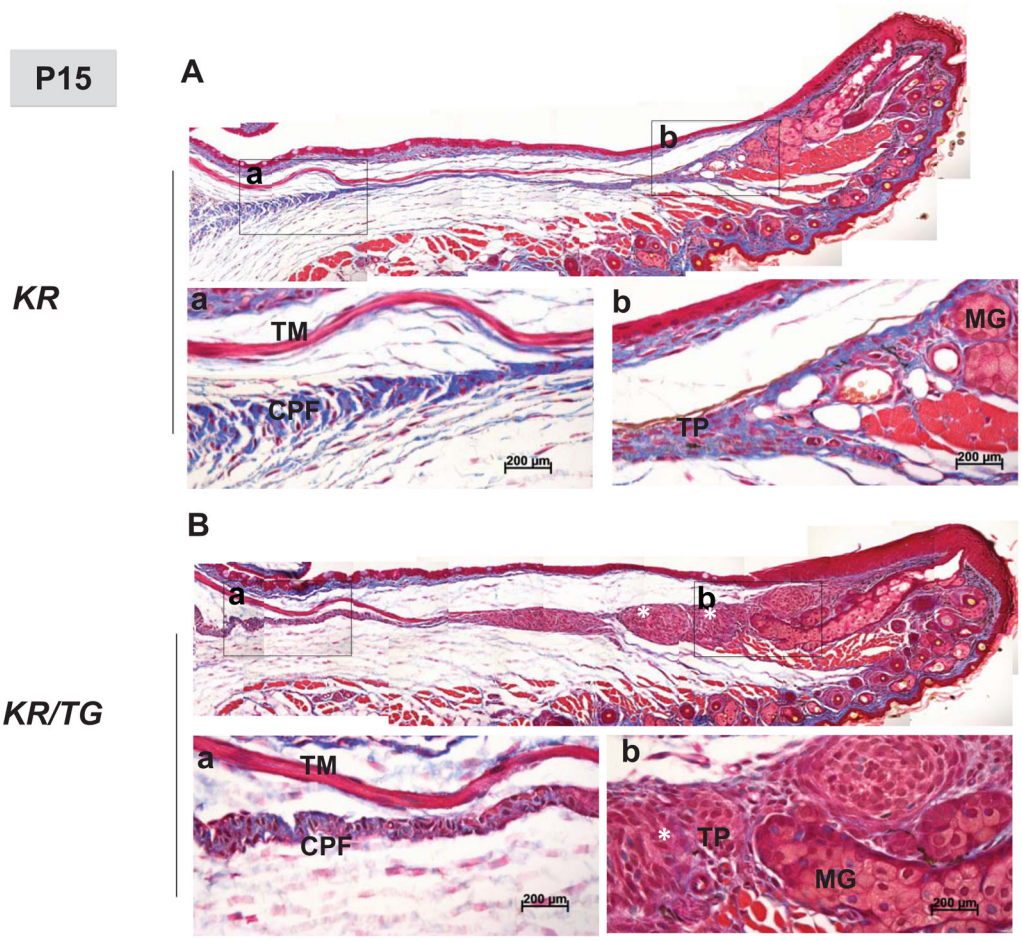

Fig. 4. Eyelid tendon and tarsal plate malformation in *KR/TG* mice

Paraffin sections of an eyelid from *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were subjected to Masson's trichrome staining. (A) A small number of fibroblasts (red colorization) and abundant extracellular matrix (blue colorization) is shown in the eyelid tendon and tarsal plate. In contrast, (B) the mutant tendons have a significant increase in the number of cells but a prominent decrease in the amount of collagen. Mutant mice also displayed cyst formation in the tarsal plate region (* in B). Higher magnification revealed normal tenocytes with elongated, spindle-shaped cells in the control mice (Aa, Ab), while compact round cells were seen in the mutant eyelid (Ba, Bb). TM, tarsal muscle; CPF, caposulopalpebral fascia; TP, tarsal plate; MG, meibomian gland.

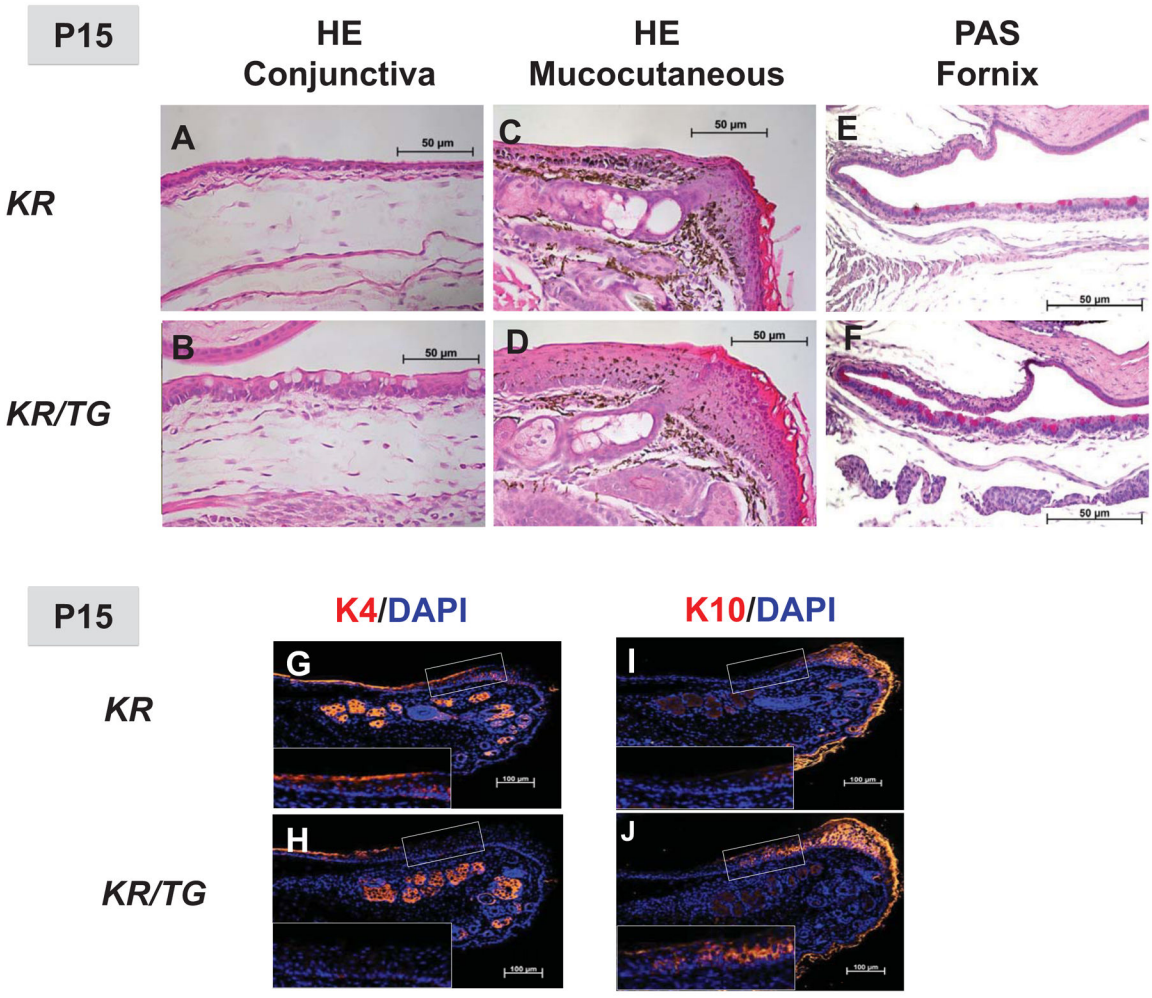

Fig. 5. Eyelid epithelium hyperplasia in *KR/TG* mice

HE staining of the palpebral epithelium showed thickening of conjunctival and mucocutaneous epithelia (B, D) compared to control (A, C). PAS reagent-stained sections revealed an increase in goblet cells at the conjunctival fornix following 15 days of TGFα induction (F). Immunofluorescence staining showed loss of K4 expression at the MCJ (H) but K10 expression was found extending over the MCJ and into the conjunctiva in mutant eyelids (J).

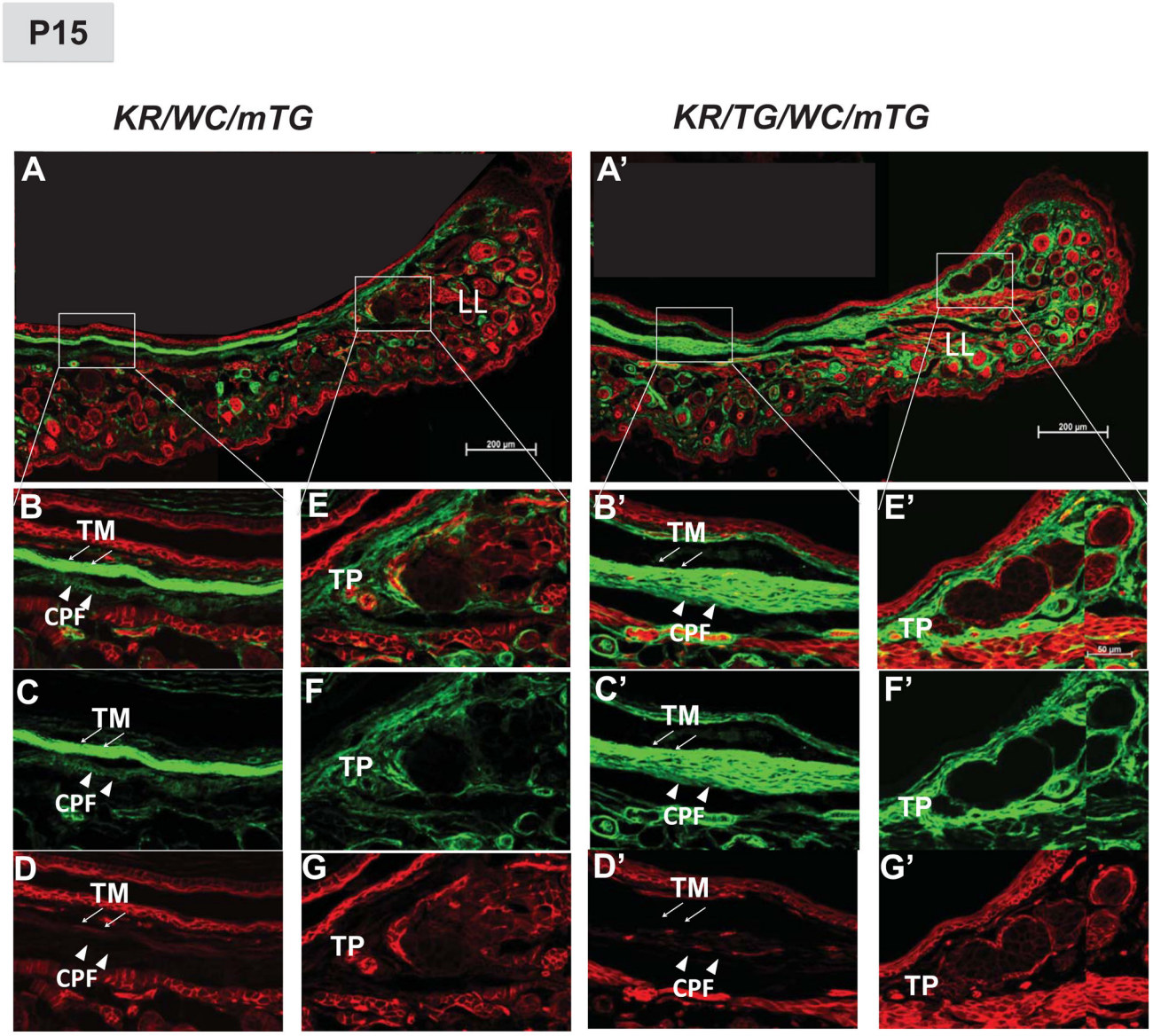

Fig. 6. The cell origin of the cyst in lower lid

KR/TG/WC/mTG quadruple transgenic mice and the *KR/WC/mTG* or *TG/WC/mTG* triple transgenic littermates, carrying a neural crest reporter gene, were induced with dox chow from P0 to P15. Cryosections (10 μm thick) were stained with DAPI. In the lower eyelid of *KR/WC/mTG* (A) mice most cells of the tendon (B–D) and tarsal plate (E–G) are green. In *KR/TG/WC/mTG* mutant mice (A′), GFP protein expression increased at the tendon CPF (arrow head in B′–D′). The tarsal plate is thickened with more cells accumulating around the meibomian gland (E′–G′). Neural crest derived tarsal muscle is barely affected by excess TGFα (arrow in B–D, B′–D′).

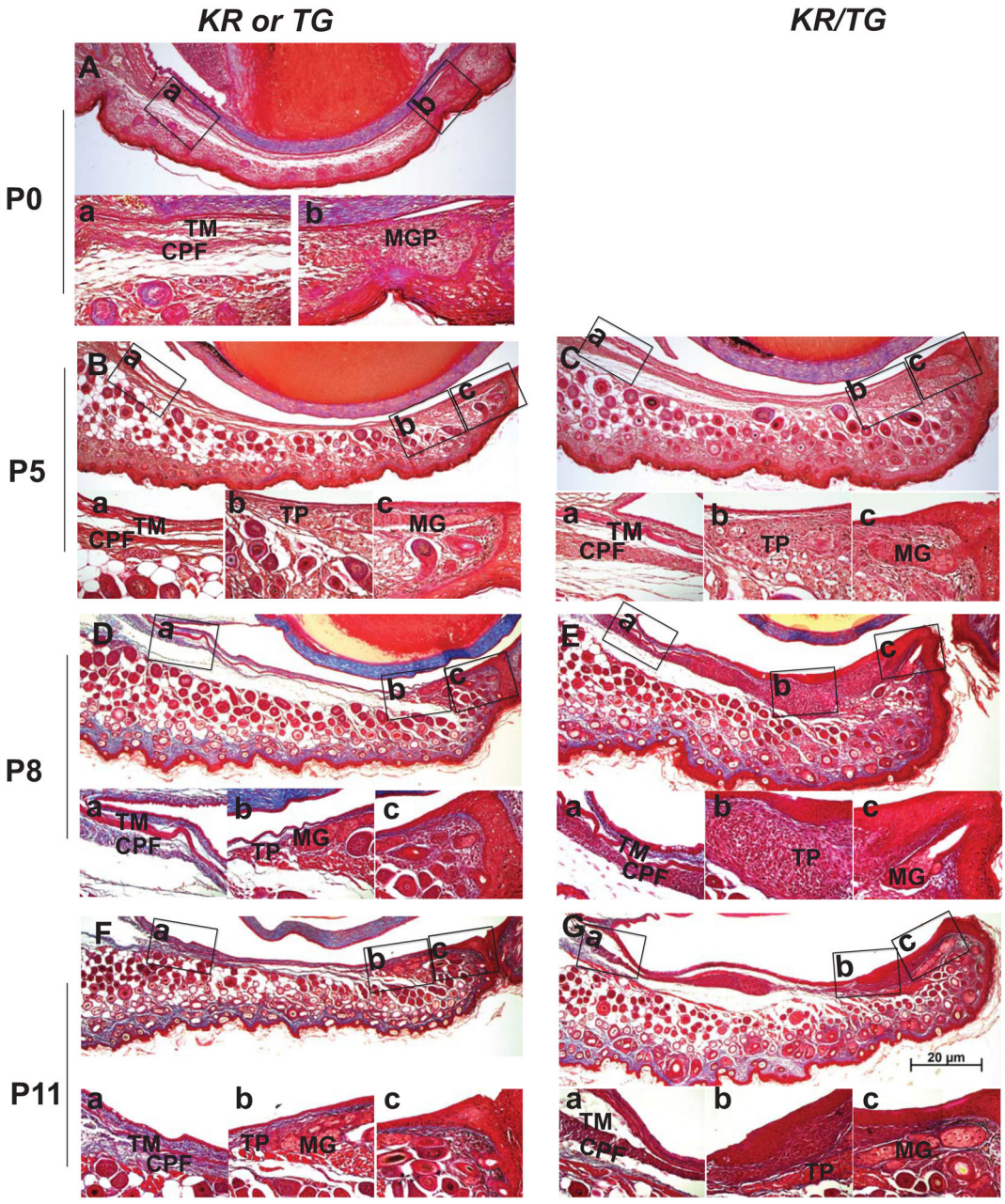

Fig. 7. Meibomian gland and tarsal plate morphogenesis in *KR/TG* and control mice

Eyes of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to different time points (P0, P5, P8, P11) were collected. Paraffin sections were subjected to Masson's trichrome staining. Masson's trichrome staining revealed the eyelid as a thin fold of fused skin at P0 (A). The primordium of the tendon (Aa) and the placode of the meibomian gland are already in place (Ab). At P5 (B), tendon (Ba) and tarsal plate primordium (Bb) forms by condensation of mesenchymal cells. The meibomian glands elongate (Bc). At P8 the meibomian gland begins to branch and invaginate into the tarsal plate (D). By P11 (F), clusters of meibomian gland acini have formed (Fb, Fc). The anomaly of CPF is first noticed at P5 (C). Abnormal accumulation of mesenchymal cells is seen in

the stroma at the eyelid tip (Cc). Meibomian gland invagination and elongation is hampered as evidenced by shorter glands (Cc). The tendon fibers are slightly thickened in comparison to control mice (Ca, Cb). Both the epidermal and conjunctival epithelium of eyelids exhibit hyperplasia. By P8, a clear distinction can be seen between the mutant and control eyelids (E). Large numbers of cells are present at the tendon region and eyelid tip (Ea, Eb). In severely affected eyes cysts form at P8 (E). The meibomian gland orifice is mislocated (Ec). At P11 (G), more mesenchymal cells condense at the eyelid tip, the tendon robustly thickens and the eyelid stroma is filled with a large number of mesenchymal cells. TM, tarsal muscle; CPF, caposulopalpebral fascia; TP, tarsal plate; MG, meibomian gland; MGP, meibomian gland primodium.

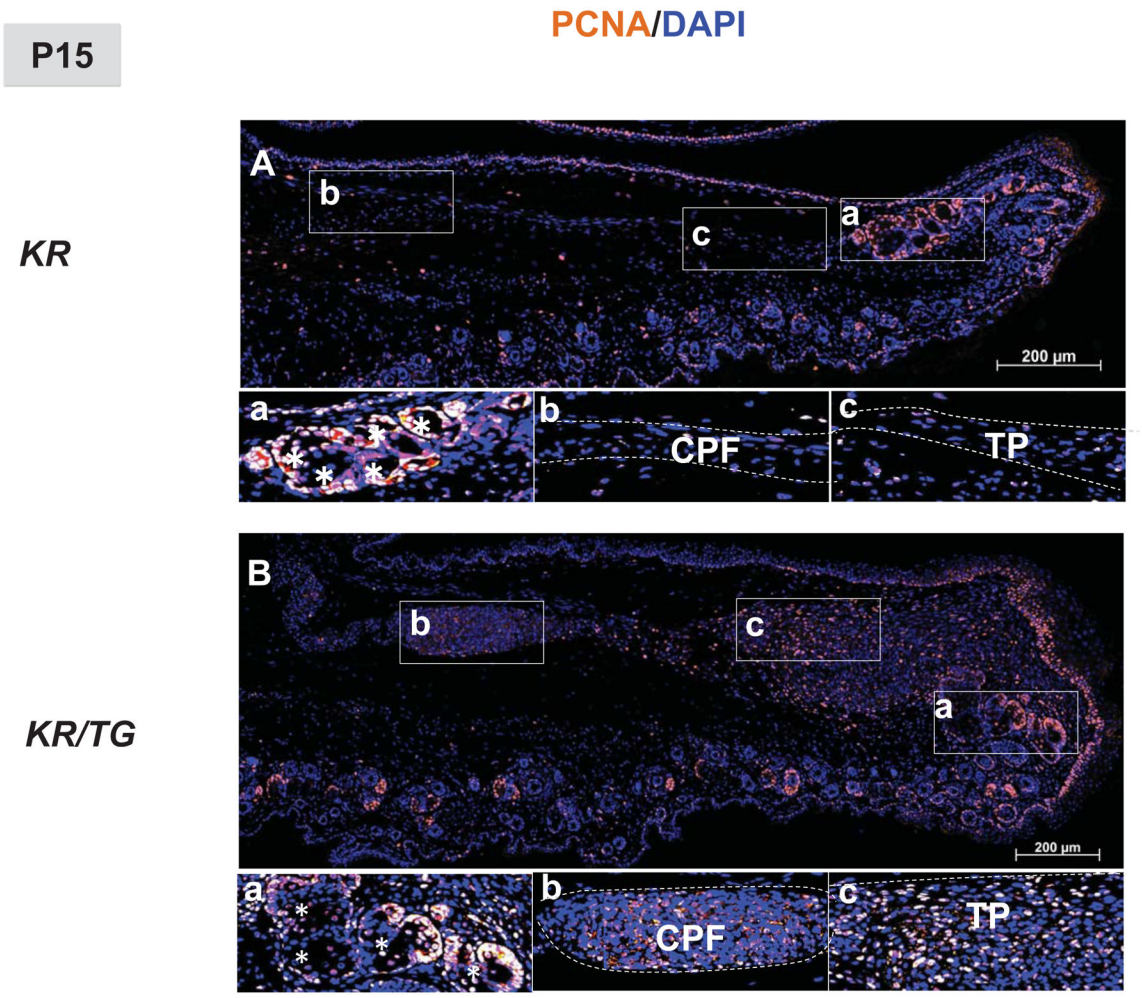

Fig. 8. Proliferation of the meibomian gland epithelial cells and the tenocytes at P15

Paraffin sections of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were subjected to immunofluorescence staining with PCNA. IF staining shows positive anti-PCNA cells distributed at the basal layer of the meibomian gland (Aa, Ba) in both transgenic and control mice. PCNA is intensely expressed in the mutant tendon (Bb) and tarsal plate (Bc) while only scattered throughout the control eyelid tendon (Ab) and tarsal plate (Ac). TM, tarsal muscle; CPF, caposulopalpebral fascia.

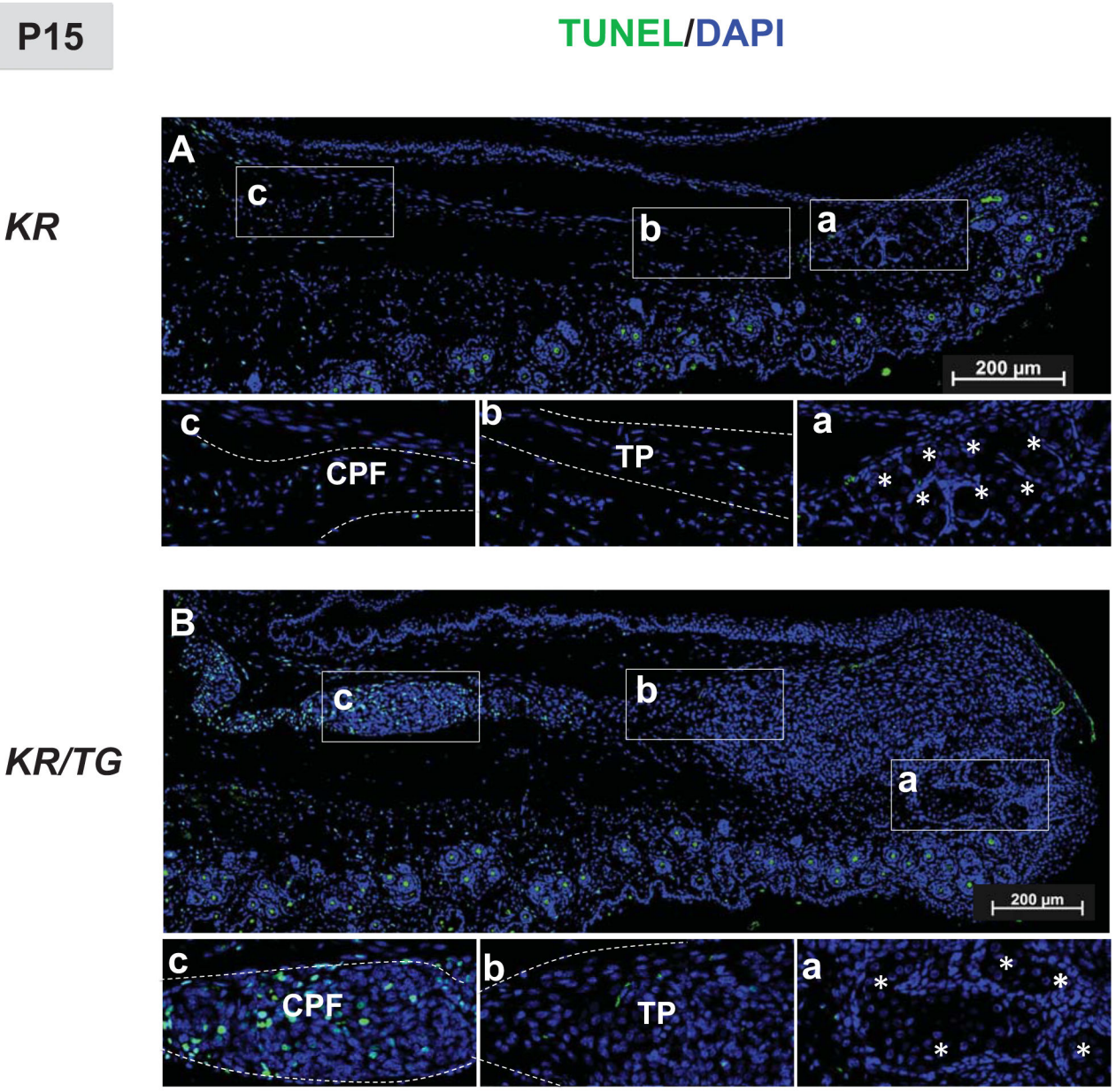

Fig. 9. Apoptosis of the meibomian gland epithelial cells and the tenocytes at P15

Paraffin sections of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were subjected to TUNEL assay. Very few cells undergo apoptosis in the acini of the meibomian glands in control mice (Aa) and bi-transgenic mice (Ba), while numerous apoptotic cells are found in the eyelid mesenchyme of bi-transgenic mice (Bb, Bc) compared to control mice (Ab, Ac). TM, tarsal muscle; CPF, caposulopalpebral fascia.

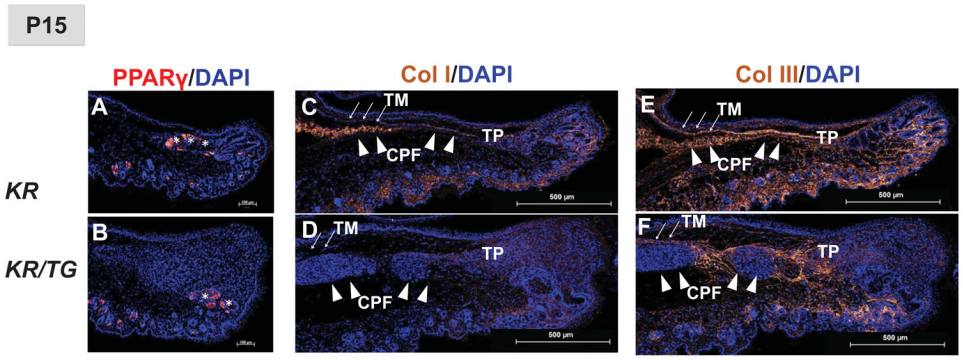

Fig. 10. Differentiation of the meibomian gland epithelial cells and the tenocytes at P15

Paraffin sections of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were subjected to immunofluorescence staining with anti-PPARγ, anti-collagen I and anti-collagen III antibody. PPARγ expression was seen in the meibomian gland acini at P15 of control mice (A). In *KR/TG* mice, though there is a defect in meibomian gland morphogenesis, the acini still expressed PPARγ (B). The extracellular matrix components collagen I (C) and collagen III (E) are rich in the control eyelid tendon and tarsal plate; however, are dramatically decreased in the eyelid stroma in mutant mice (D, E). TM, tarsal muscle (arrows); CPF, caposulopalpebral fascia (arrowhead).

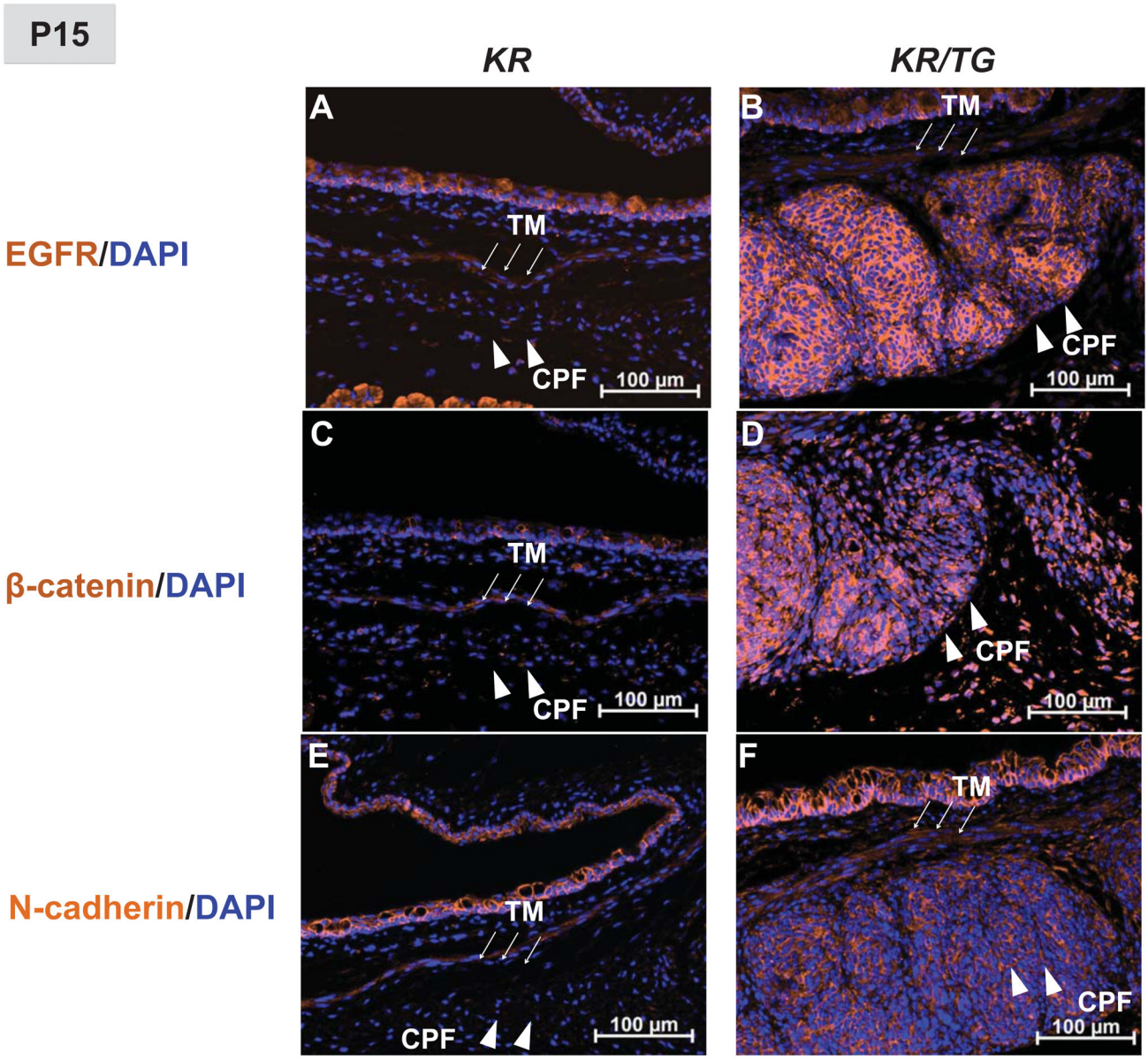

Fig. 11. Expression of EGFR, β-catenin and N-cadherin in the tenocytes of *KR/TGFα* mice eyelid

Paraffin sections of *KR/TG* bi-transgenic mice and single-transgenic littermates upon dox induction from P0 to P15 were subjected to immunofluorescence staining with anti-EGFR, anti-β-catenin and anti-N-cadherin antibody. In control eyelid tendon at P15, there is little, if any, EGFR expression (A). In the mutant eyelid the expression of EGFR (B) is dramatically enhanced by TGFα stimulation. IF staining also revealed enhanced β-catenin expression and nuclear translocation (D) in the mutant tendon. N-cadherin is strongly expressed in the tenocytes of *KR/TG* mice (F) compared to control mice (E). TM, tarsal muscle (arrows); CPF, caposulopalpebral fascia (arrowheads).