

Ectodysplasin A protein promotes corneal epithelial cell proliferation

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The *EDA* gene encodes ectodysplasin A (Eda), which if mutated causes X-linked hypohidrotic ectodermal dysplasia (XLHED) disease in humans. Ocular surface changes occur in XLHED patients whereas its underlying mechanism remains elusive. In this study, we found Eda was highly expressed in meibomian glands, and it was detected in human tears but not serum. Corneal epithelial integrity was defective and the thickness was reduced in the early postnatal stage of *Eda* mutant *Tabby* mice. Corneal epithelial cell proliferation decreased and the epithelial wound healing was delayed in *Tabby* mice, whereas it was restored by exogenous Eda. Eda exposure promoted mouse corneal epithelial wound healing during organ culture, whereas scratch wound assay showed that it did not affect human corneal epithelial cell line migration. Epidermal growth factor receptor (EGFR), phosphorylated EGFR (p-EGFR), and phosphorylated ERK1/2 (p-ERK) were down-regulated in *Tabby* mice corneal epithelium. Eda treatment up-regulated the expression of Ki67, EGFR, p-EGFR, and p-ERK in human corneal epithelial cells in a dose-dependent manner. In conclusion, Eda protein can be secreted from meibomian glands and promotes corneal epithelial cell proliferation through regulation of the EGFR signaling pathway. Eda release into the tears plays an essential role in the maintenance of corneal epithelial homeostasis.

Ectodysplasin A (Eda)³ is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. It

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³ The abbreviations used are: Eda, ectodysplasin A; Edar, ectodysplasin A receptor; XLHED, X-linked hypohidrotic ectodermal dysplasia; MGD, meibomian gland dysfunction; qRT, quantitative RT; EdU, 5-ethynyl-2'-deoxyuridine; HCE, human corneal epithelial; CCK8, cell counting kit 8.

contains a short N-terminal intracellular domain, transmembrane region, and extracellular portion with collagenous domain and a TNF-ligand motif in its C-terminal region (1, 2). Cleavage of Eda by the furin-like enzyme leads to formation of soluble extracellular molecule, which is able to interact with its Eda receptor (Edar) and mediate downstream signals (3, 4). In 1996, the *EDA* gene was first identified and a mutant form was found to be responsible for X-linked hypohidrotic ectodermal dysplasia (XLHED), the most common genetic disorder of ectodermal development in human beings (5). Subsequently, numerous studies determined the function of *EDA* expression on development of ectodermal structures such as teeth, hair, and several exocrine glands including sweat glands and meibomian glands (6). Intensive Eda-Edar-linked signal transduction pathway characterization showed that the Wnt, Shh, BMP, and lymphotoxin- β (LT β) pathways (7–9) mediate hair as well as other ectodermal tissue development rather than the well documented nuclear factor κ B (NF- κ B) signaling pathway (10–14).

Along with the corneal epithelial defect in this *EDA* mutant, meibomian gland development is severely retarded in XLHED patients. Ocular surface abnormalities such as dry eye, chronic conjunctivitis, and blepharitis become evident during the early childhood of these patients and gradually progresses with age (15, 16). Other manifestations of this condition include corneal pannus formation, which significantly reduces the visual acuity and the life quality of these patients over the long run (17). In addition, Cui *et al.* (18) demonstrated progressive development of corneal lesions such as neovascularization, keratitis, ulceration, and keratinization in *Eda* mutant *Tabby* mice (18). Our recent study also showed that pathological ocular surface changes in 6–8-week-old *Tabby* mice resembled those described in meibomian gland dysfunctional evaporative dry eye patients (19). Interestingly, we found an earlier report in which ectopic *Eda* gene expression in *Tabby* mice rescued to a large extent ocular surface abnormalities, despite absence of meibomian gland development in the *EDA* gene knock-in mice (18). These results indicate that *EDA* may instead support ocular surface integrity expression rather than promote meibomian gland development.

Eda mRNA is expressed in various organs and tissues, including heart, kidney, pancreas, brain, lung, liver, skeletal muscle, teeth, as well as the skin during both embryonic development and adulthood (4, 5). Mouse corneal and conjunctival

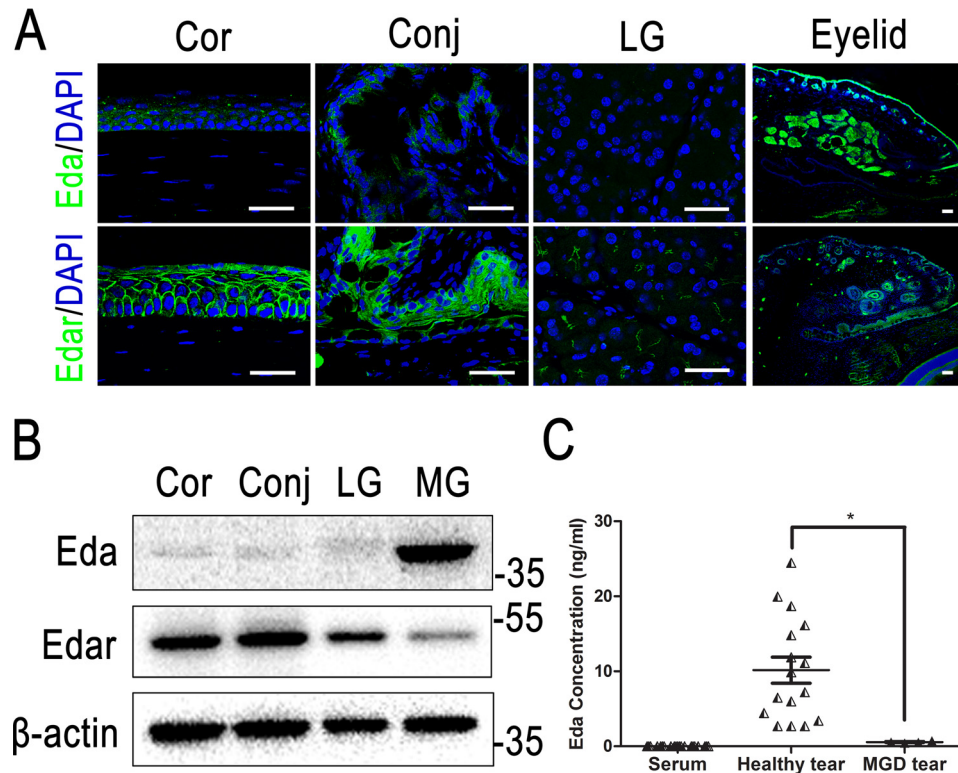


Figure 1. Meibomian gland secretes Eda protein. *A*, immunofluorescent staining showed Eda was weakly expressed in the corneal (*Cor*) and conjunctival (*Conj*) epithelium and lacrimal gland (*LG*), whereas it was strongly expressed in the meibomian gland (*MG*). Edar was highly expressed in the corneal and conjunctival epithelium, whereas it was weak in the lacrimal gland and meibomian gland. *B*, Western blot results showed similar expression patterns of Eda and Edar in corneal epithelium, conjunctiva, lacrimal gland, and meibomian gland tissues. *C*, ELISA results showed that Eda was undetectable in normal human serum, Eda average protein concentration in healthy human tears was 10.16 ± 1.73 ng/ml, whereas it was 0.57 ± 0.07 ng/ml in tears from MGD patients (*, $p < 0.05$). Scale bars represent $50 \mu\text{m}$.

tissue both express *Eda* and *Edar* mRNA (18). However, meibomian gland Eda expression is still unknown. In our current study, we found that Eda protein is secreted from the meibomian glands into the tears and regulates corneal epithelial cell proliferation through activating the EGFR signaling pathway. We deal here with the significance of these findings for possible use in a clinical setting.

Results

Meibomian gland secretes Eda protein

Meibomian gland development is dependent on *Eda* gene expression because in *Tabby* mice, a natural *Eda* mutation resulted in no meibomian gland morphogenesis (18). However, the contribution made by the Eda protein to postnatal ocular surface tissue remains unknown. Immunofluorescent staining detected strong Eda protein expression in the meibomian gland but very weak expression in the corneal and conjunctival epithelium, as well as lacrimal gland. On the other hand, Edar was highly expressed in both the corneal and conjunctival epithelium but weakly expressed in the lacrimal gland and meibomian glands (Fig. 1*A*). Western blot analysis confirmed differences in corneal, conjunctival, lacrimal gland, and meibomian gland Eda and Edar expression patterns (Fig. 1*B*). Based on Eda being a secretory protein, we proposed that Eda protein could be secreted from the meibomian glands into the tears and targeted to the ocular surface epithelium. Accordingly, tear sample Eda protein content was measured in 16 healthy individuals and 4

meibomian gland dysfunction (MGD) patients. Its content was also measured in serum samples from 21 healthy individuals. ELISA results showed that the average Eda protein concentration in healthy human tears was 10.16 ± 1.73 ng/ml, whereas it was only 0.57 ± 0.07 ng/ml in tears from MGD patients and undetectable in normal human serum (Fig. 1*C*). Taken together, these results indicate that the meibomian gland, but not lacrimal gland, is essentially the major source of Eda in the tears.

Corneal epithelial cell proliferation decrease in *Tabby* mice

The corneal epithelium is composed of non-keratinized, stratified squamous epithelium and it is about 4 to 6 cell layers thick in healthy adult mice. Our recent study revealed that corneal abnormalities in *Tabby* mice appeared as early as postnatal 4 week and the severity of the pathological changes increased with age (19). As described in our previous study, corneal epithelial defects became evident in 4-week-old *Tabby* mice based on sporadic sodium fluorescein staining (Fig. 2*A*), indicating compromised corneal epithelial barrier function in *Tabby* mice. H&E staining of *Tabby* mice corneal tissue revealed mild central corneal epithelium thinning compared with their wild-type littermates at 4 or 8 weeks of age, and the corneal stromal thickness was also reduced in *Tabby* mice (Fig. 2*B*).

To determine the proliferation status of corneal epithelium in *Tabby* mice, we measured their *Ki67* gene expression. Quantitative RT (qRT)-PCR results indicated that *Ki67* mRNA was

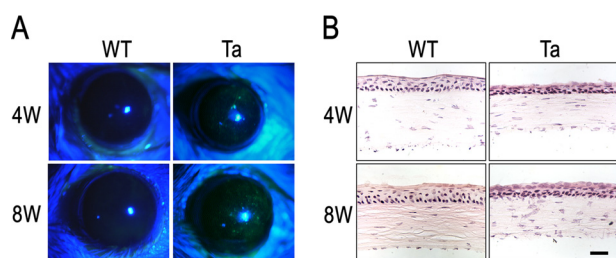


Figure 2. Corneal epithelial defect in *Tabby* mice. *A*, sodium fluorescein staining showed sporadic positive staining of the corneal epithelium in 4-week-old, *Tabby* mice (*Ta*), which increased in 8-week-old *Tabby* mice, whereas there was no fluorescein staining in the wild-type littermates (*WT*). *B*, H&E staining of corneal tissue showed that the thickness of the central corneal epithelium was 4 to 5 layers in wild-type mice, whereas it fell to 3 to 4 layers in *Tabby* mice. Scale bar represents 50 μ m.

down-regulated in 4- and 8-week-old *Tabby* mice corneal epithelium compared with their wild-type littermates (Fig. 3A). Western blot analysis results confirmed that Ki67 protein expression was also less in both 4- and 8-week-old *Tabby* mice corneal epithelium (Fig. 3B). Whole mount corneal epithelial Ki67 immunostaining showed that Ki67 positive cells were mainly located in the corneal epithelial basal layer (Fig. 3C). There were fewer Ki67 positive cells in the central basal corneal epithelial layer of 8-week-old *Tabby* mice than in the wild-type littermates (Fig. 3D). We also tested the Ki67 expression level on conjunctival tissue. Real-time PCR, Western blot analysis, and immunostaining of Ki67 results all indicated lower expression of Ki67 in *Tabby* mice when compared with wild-type littermates (supplemental Fig. S1).

We further performed EdU injection to label the proliferating epithelial cells. Anti-EdU antibody immunostaining showed that EdU-positive cells were located in the basal layer of the corneal epithelium (Fig. 3E), and there were fewer EdU-positive cells in *Tabby* mice than in the wild-type littermates (Fig. 3F). Collectively, these data indicate that corneal epithelial proliferation was reduced in *Tabby* mice compared with their wild-type littermates.

Corneal epithelial wound healing delay in *Tabby* mice

A 2.0-mm diameter corneal epithelial wound induced by debridement required only 24 h for complete healing to occur in wild-type mice, however, in 8-week-old *Tabby* mice the defect was not completely resurfaced even 36 h later (Fig. 4A). The wound closure extent at 12 h was 48.9% in the *Tabby* mice and 59.1% in the wild-type mice, whereas at 24 h it was 67.5% in the *Tabby* mice and 99.3% in the wild-type mice (Fig. 4C). *Tabby* mouse corneal epithelial wound healing at both time points was significantly delayed.

We further determined if Eda treatment could promote corneal epithelial wound healing in *Tabby* mice. A 2.5-mm diameter corneal epithelial debridement wound was created on the central corneas of 6-week-old *Tabby* mice, 5 μ l of 100 ng/ml of mouse recombinant Eda protein (191-ED, R&D Systems) in PBS was applied in their conjunctival sac four times per day. PBS was used as a control (Fig. 4B). The results showed that epithelial wound closure was faster at 12, 24, and 36 h post-wounding after application of Eda (Fig. 4D), indicating exogenous Eda could reverse the epithelial wound healing delay.

To investigate whether exogenous Eda can rescue the corneal epithelial defect in *Tabby* mice, the same dosage of Eda protein was applied for 3 weeks in 4-week-old *Tabby* mice. Slit-lamp microscopy images showed scabrous corneal surface reflection in *Tabby* mice treated with PBS for 3 weeks, fluorescein staining showed punctate corneal staining in the corneal epithelium. However, *Tabby* mice treated with topical Eda protein for 3 weeks showed smooth corneal surface and negative fluorescein staining (Fig. 5A). H&E staining showed more condensed corneal epithelium and smooth epithelial surface in *Tabby* mice after Eda treatment (Fig. 5B). Ki67 immunostaining showed increased positive epithelial cells in *Tabby* mice treated with Eda (Fig. 5C). Cell counting results confirmed Ki67-positive corneal epithelial cells were significantly increased in *Tabby* mice after Eda treatment (Fig. 5D). These data indicated that exogenous Eda can rescue the *Tabby* mice corneal epithelium to a normal status, and promote proliferation of corneal epithelial cells in *Tabby* mice.

Because meibomian gland secretions are a complex mixture containing various proteins and lipid components, one may argue that other components besides Eda may also affect corneal epithelial wound healing. To delineate the contribution made by Eda to the wound healing response, 8-week-old wild-type mice eyeballs that had a 2.0-mm corneal epithelial debridement wound on the central cornea were cultured in DMEM + 2% FBS in the absence or presence of 20 ng/ml of mouse recombinant Eda protein or 10 ng/ml of mouse recombinant EGF protein (PMG8041, Life Technologies) for different durations from 12 to 36 h. Sodium fluorescein staining evaluated the wound closure process during these periods (Fig. 6A). After 12 h of culture, 26.5% of the surface area in the Eda-treated group was healed, whereas 28.7% of the wound was closed in the EGF-treated group and only 9.6% in the control group. At 24 h, the wound closure reached 80.0% in the Eda-treated group, 100% in the EGF-treated group and 58.7% in the control group. At 36 h, the epithelial defect was completely healed in both the Eda-treated and control groups (Fig. 6B). H&E staining revealed that there was only one layer of epithelial cells in the central cornea of the control eyeballs, whereas there were two to three layers of epithelial cells in the EGF- and Eda-treated corneas after 36 h culture (Fig. 6C). These results validate that Eda protein expression in the tears promotes corneal epithelial wound healing.

Eda has no effect on HCE cell migration

To further clarify whether Eda-induced stimulation of corneal epithelial wound healing is due to increases in cell proliferation or migration, or both responses, we performed a scratch wound healing assay using HCE cells cultured in different concentrations of human recombinant Eda (0, 5, 10, and 20 ng/ml) (Fig. 7A). The wound closure rates showed no significant difference between different groups at 20 h after scratching (Fig. 7B), indicating that Eda did not promote HCE cell migration.

Eda promotes corneal epithelial proliferation through EGF-EGFR signaling pathway

In the corneal epithelium, EGFR-mediated responses are essential for maintaining epithelial homeostasis (20). For

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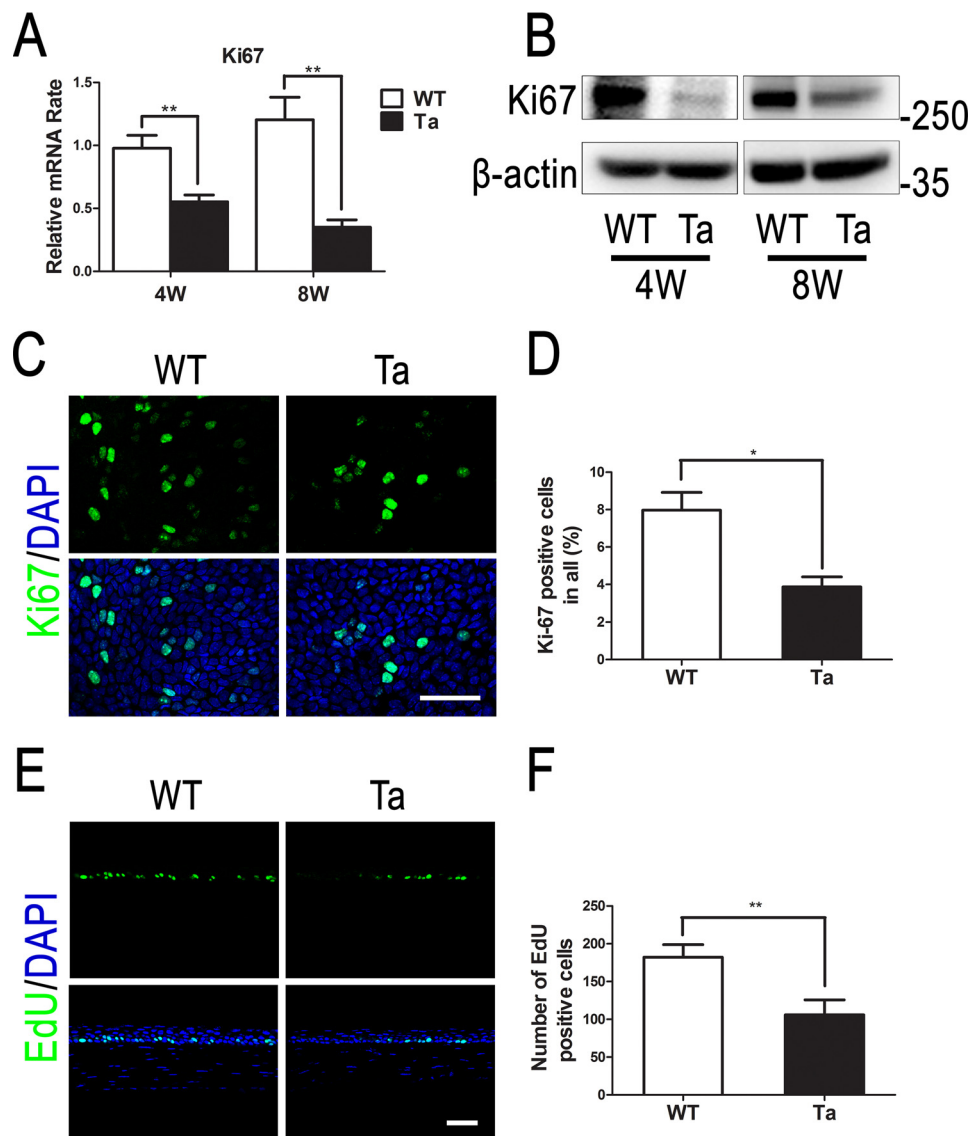


Figure 3. Corneal epithelial cell proliferation decrease in *Tabby* mice. *A*, qRT-PCR results indicated lower *Ki67* mRNA expression in 4- and 8-week-old *Tabby* mice corneal epithelium ($n = 5$, **, $p < 0.01$). *B*, Western blot analysis showed that *Ki67* expression decreased in 4- and 8-week-old *Tabby* mice corneal epithelium. *C*, whole mount immunostaining of *Ki67* showed sporadic positive cells in the basal layer of the corneal epithelium in both 8-week-old wild-type mice and *Tabby* mice. *D*, cell counting results showed less *Ki67*-positive cells in the central basal corneal epithelia of *Tabby* mice when compared with the wild-type littermates ($n = 3$, *, $p < 0.05$). *E*, EdU staining after 48 h chasing showed that EdU-positive cells were located in the basal layer of the corneal epithelium in both wild-type mice and *Tabby* mice. *F*, cell counting showed less EdU-positive cells in *Tabby* mice corneal epithelium compared with those in the wild-type littermates ($n = 3$, **, $p < 0.01$). Scale bars represent 50 μm .

instance, EGF ligand-mediated activation of EGFR accelerates corneal epithelial wound healing (21). A previous report (22) has shown that EGFR is down-regulated in skin tissue of XLHED patients and *Tabby* mice. We then proposed that there is cross-talk between the EDA-EDAR signaling pathway and EGF-EGFR signaling in the corneal epithelium. qRT-PCR results showed *EGFR* mRNA expression was lower in the corneal epithelium of 8-week-old *Tabby* mice compared with that in their littermates (Fig. 8A). Immunofluorescent staining also showed that EGFR expression was much weaker in corneal epithelium of *Tabby* mice compared with wild-type mice, especially in the basal layer (Fig. 8B). Western blot analysis confirmed both total EGFR and *p*-EGFR were both down-regulated in *Tabby* mice. Moreover, *p*-ERK, a mediator of the EGF-EGFR signaling pathway, was also down-regulated in *Tabby* mice (Fig.

8C). These results confirmed that the EGF-EGFR signaling pathway is down-regulated in *Eda* mutant *Tabby* mice.

We further validated the effect of Eda on HCE cell proliferation with the CCK8 assay. The results showed that Eda promoted HCE cell proliferation in a dose-dependent manner at concentrations of 5 to 10 ng/ml and reached a plateau at 20 ng/ml (Fig. 9A). qRT-PCR analysis showed that *EGFR* mRNA was up-regulated in HCE cells after exposure to either 5 or 10 ng/ml of Eda for 4 h (Fig. 9B). After 24 h treatment with different concentrations of Eda, HCE cells were harvested for Western blot analysis. The results showed that Eda treatment up-regulated the expression of *Ki67*, EGFR, *p*-EGFR, and *p*-ERK in a dose-dependent manner (Fig. 9C). We also performed EGFR immunostaining and found that EGFR expression was obviously increased in the cornea of *Tabby* mice after 3 weeks treat-

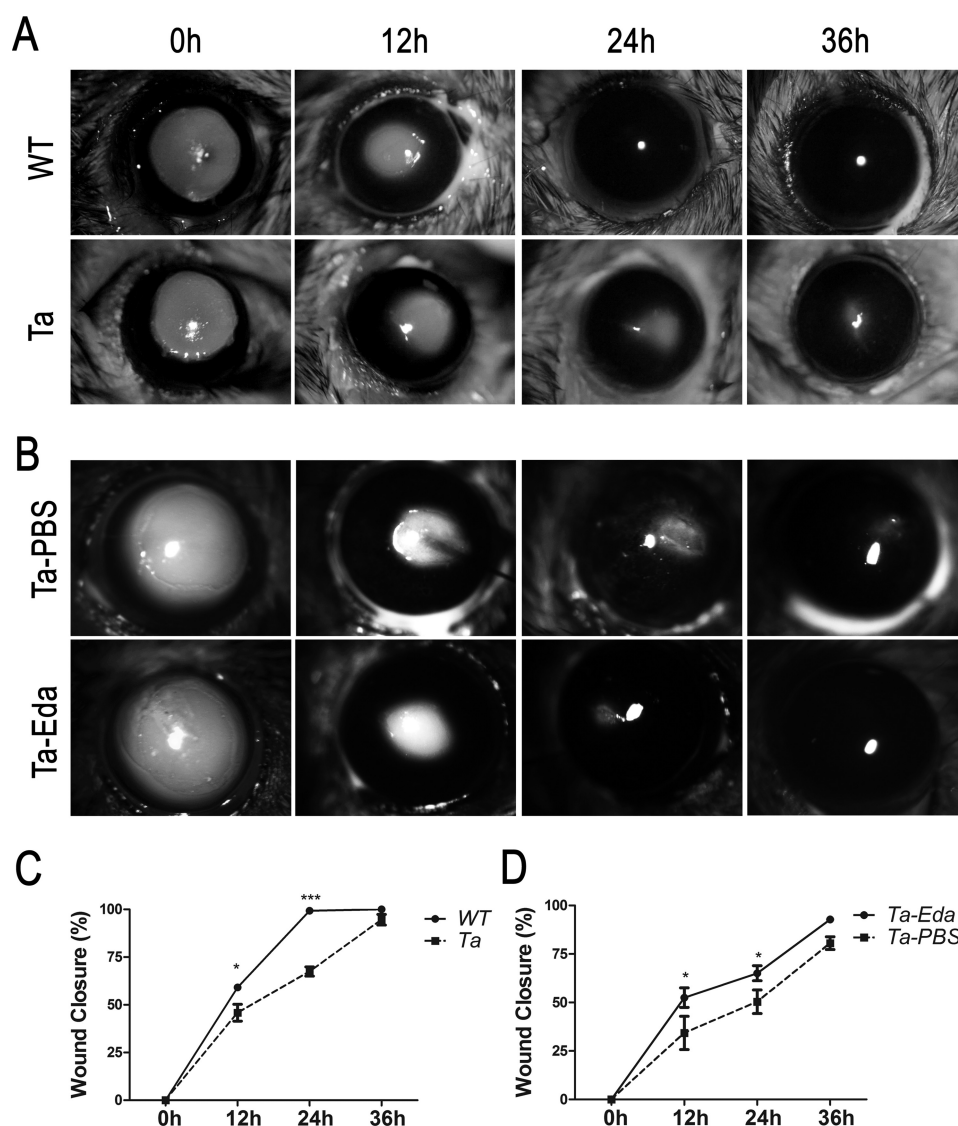


Figure 4. Corneal epithelial wound healing delayed in *Tabby* mice and promoted by exogenous Eda. *A*, 2.0-mm central corneal epithelial debridement was performed in 8-week-old wild-type mice and *Tabby* mice. Sodium fluorescein staining showed gradual wound closure. The corneal epithelium was almost completely healed in wild-type mice at 24 h after wounding, whereas the epithelial defect was not completely resurfaced even at 36 h after the wounding in *Tabby* mice. *B*, 2.5-mm central corneal epithelial debridement wound was created in 6-week-old *Tabby* mice, 100 ng/ml of mouse recombinant Eda protein in PBS was applied in the conjunctival sac of *Tabby* mice, four times per day. PBS alone was used as control. Sodium fluorescein staining showed that the epithelial wound was not healed at 36 h in the PBS group, whereas was almost completely healed in the Eda-treated group. *C*, for the epithelial wound healing in *Tabby* mice and wild-type mice, the extent of the wound closure rate at 12 h was 48.9% in *Tabby* mice and 59.1% in the wild-type mice. It was 67.5% in the *Tabby* mice and 99.3% in the wild-type mice at 24 h. *Tabby* mice showed significant delayed corneal wound healing at both time points ($n = 4$, *, $p < 0.05$; ***, $p < 0.001$). *D*, for the epithelial wound healing in *Tabby* mice with or without Eda treatment, extent of the wound closure rate at 12 h was 52.5% in the Eda-treated group (*Ta-Eda*) and 34.3% in *Tabby* mice treated with PBS alone (*Ta-PBS*). It was 65.1% in *Ta-Eda* and 50.4% in *Ta-PBS* at 24 h, 92.8% in *Ta-Eda* and 80.6% in *Ta-PBS* at 36 h. Eda treatment significantly promoted wound healing in *Tabby* mice at all the time points ($n = 4$, *, $p < 0.05$).

ment with topical Eda, whereas it remained at a low level in PBS-treated cornea (Fig. 9D). Taken together, these results indicate that Eda promotes corneal epithelial cells proliferation through activation of the EGF-EGFR signaling pathway.

Discussion

Tear production and its release onto the anterior ocular surface are essential for maintaining epithelial integrity and functional activity. These surface attributes are sustained by a complex mixture of components produced by the lacrimal glands, meibomian glands, and ocular surface epithelium. They maintain corneal transparency, lubricate the ocular surface, support anti-pathogenic infiltration, protect against tissue destruction,

and promote epithelial renewal (23). These homeostatic maintaining components are dispersed within the tear film lipid, aqueous, and mucin layers (24). The lipid layer originates from the meibomian glands and forms the superficial layer of the tear film, which reduces tear film evaporative losses (25). The realization of the importance of the lipid layer to maintaining tear film homeostasis and ocular surface health made it apparent that the main cause of evaporative dry eye disease is meibomian gland dysfunction. In numerous studies, it was shown that this condition led to tear film lipid deficiency (26, 27). However, there are very few studies describing any roles provided by meibomian gland secretory proteins in maintaining ocular surface health even though in humans, proteomic analysis identified

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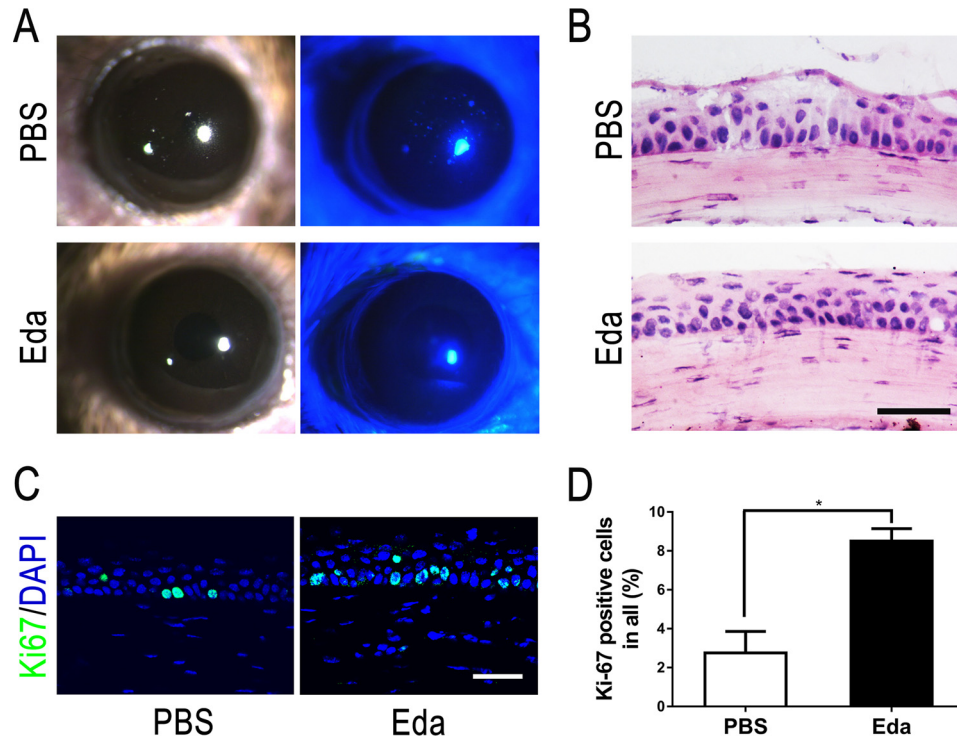


Figure 5. Exogenous Eda rescues corneal epithelial defect in *Tabby* mice. *A*, scabrous corneal surface reflection was present in *Tabby* mice treated with PBS for 3 weeks, fluorescein staining showed punctate corneal staining in the corneal epithelium. However, *Tabby* mice treated with topical Eda protein for 3 weeks showed smooth corneal surface and negative fluorescein staining. *B*, H&E staining showed rough corneal epithelial surface in PBS-treated *Tabby* mice, whereas smooth surface and condensed corneal epithelium in *Tabby* mice after Eda treatment. *C*, immunostaining of Ki67 showed increased positive epithelial cells in *Tabby* mice treated with Eda. *D*, cell counting results confirmed that Ki67-positive cells significantly increased in *Tabby* mice corneal epithelium after Eda treatment ($n = 4$, **, $p < 0.01$). Scale bar represents 50 μm .

more than 90 proteins secreted from meibomian glands (28). In the current study, we show for the first time that meibomian glands express and secrete Eda, which plays an important function in promoting corneal epithelial cell proliferation and maintaining epithelial integrity.

Immunostaining and Western blot analysis clearly demonstrated that Eda protein is mainly produced by meibomian glands, whereas Edar is mainly located on corneal and conjunctival epithelial cell membranes. ELISA showed that the Eda concentration was much higher in tears from healthy subjects than those from MGD patients. These results further supported our hypothesis that Eda is produced by meibomian glands rather than lacrimal glands. On the other hand, Eda was undetectable in human serum indicating that Eda elicits its regulatory effect through a paracrine rather than a systemic mechanism. The mature soluble endogenous Eda form contains a heparan sulfate proteoglycan-binding domain, whose interaction with an extracellular matrix restricts its diffusion. On the other hand, this form is conducive for local enrichment after enzymatic mediated cell membrane Eda shedding, which fine tunes its effects (29). There is abundant heparan sulfate proteoglycan in the corneal epithelium and tears (30), which may facilitate Eda membrane binding and promote its ocular surface biological function.

We found Edar is highly expressed in the corneal and conjunctival epithelium, but not in stromal cells, indicating that Eda targeting is limited to the ocular surface epithelial cells. Several lines of evidence in our study indicate that Eda stimulates proliferation of the corneal epithelium. First, the corneal

epithelium in *Tabby* mice was thinner than in their wild-type littermates. Second, there were fewer Ki67 positive and EdU labeling cells in the basal epithelium. Third, injury-induced corneal epithelial wound healing was delayed, whereas exogenous Eda overcame prolongation of this response. Fourth, recombinant Eda protein supplementation rescued corneal epithelial defect in *Tabby* mice, and promoted corneal epithelial wound healing during *ex vivo* organ culture and in cultured HCE cells. Fifth, in contrast to its stimulation of cell proliferation, Eda protein did not hasten HCE cell migration.

We also noticed that Ki67-positive cells were mainly present in the basal layer of corneal epithelium, therefore Eda may target basal epithelial cells to regulate cell proliferation. Although *in vivo* and *ex vivo* wound healing models and cell culture experiments all showed that Eda promotes corneal epithelial cell proliferation, currently there is no direct evidence showing that Eda could access to the basal layer of the corneal epithelium so as to regulate epithelial cell proliferation. It is well known that the intercellular barrier in corneal epithelium can prevent pathogens or large molecules from entering the basal epithelium. We presume Eda may get access to the basal epithelium through other mechanisms. This mechanism may be also applied to other proteins such as EGF, because EGF is also abundant in tear film, and also plays an important role in corneal epithelial proliferation. Similar to Edar, EGF receptor (EGFR) is also expressed in the full thickness of corneal epithelium. Further study is needed to illustrate the mechanism through which Eda protein can access to the basal corneal epithelial cells.

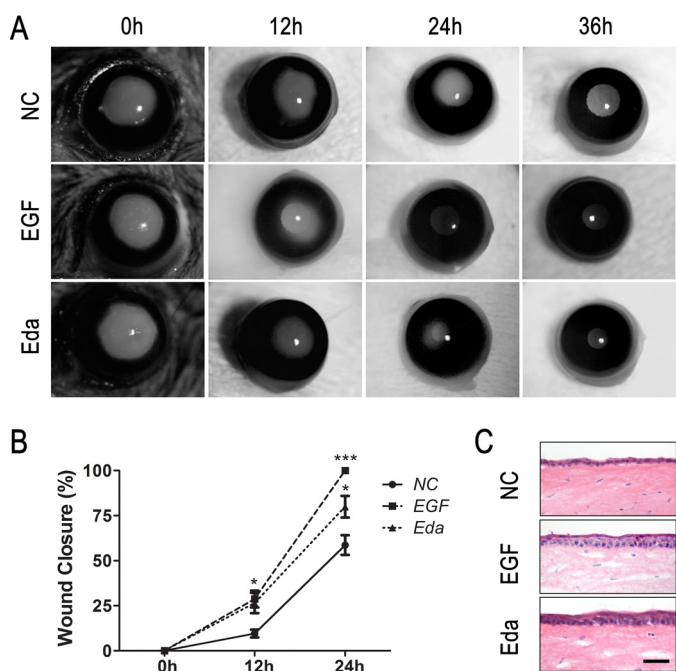


Figure 6. Eda promotes corneal epithelial wound healing during *in vitro* organ culture. A, 8-week-old wild-type mice eyeballs with a 2.0-mm corneal epithelial debridement wound on the central cornea were cultured in DMEM + 2% FBS in the absence or presence of 20 ng/ml of recombinant Eda protein or 10 ng/ml of EGF protein for different durations from 12 to 36 h. Fluorescein staining evaluated differences in wound closure extent during the culture. B, the wound closure extent reached 26.5% in the Eda treatment group (*Eda*), 28.7% in the EGF treatment group (*EGF*), whereas it was 9.6% in the control group (NC) after 12 h culture. At 24 h, the wounds had healed to 80.0% in the Eda treatment group, whereas it was 58.7% in the control group, and completed healed in the EGF group. At 36 h, the epithelial defect was completely healed in both groups ($n = 4$, $*$, $p < 0.05$). C, after a 36-h culture, H&E staining of the eyeballs showed there was a single layer of epithelial cells present in the central cornea of the control eyeballs, whereas there were two to three layers of epithelial cells in the EGF or Eda-treated corneas. Scale bar represents 50 μm .

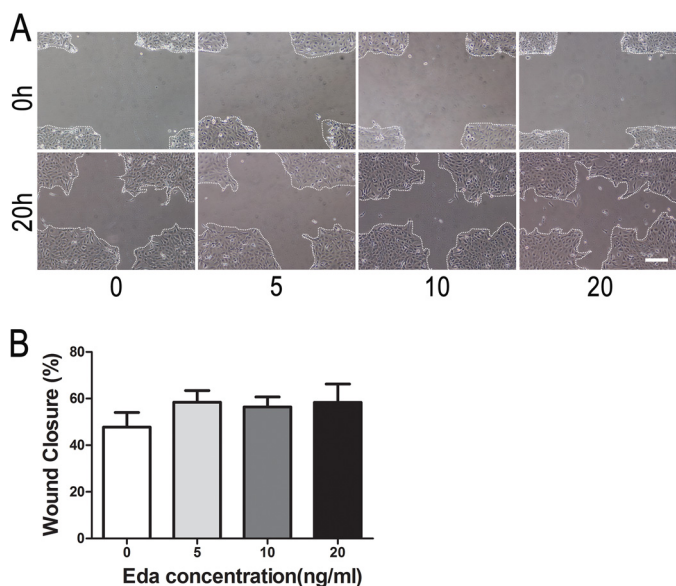


Figure 7. Negative effect of Eda on human corneal epithelial cell migration. A, HCE cells were cultured with different concentrations of Eda (0, 5, 10, and 20 ng/ml). Scratch wound healing assay was performed after the cells reached confluence. The wound surface area at 0 and 20 h after scratching was documented. l represents 100 μm . B, the wound closure extent was invariant between different groups ($n = 4$).

In previous studies, it was shown that Eda promoted epithelial cell proliferation through the NF- κ B signaling pathway in mammary glands (31). On the other hand, exposure to recombinant Fc-Eda A1 protein up-regulated EGFR agonists, Epigen (*Epgn*) and Amphiregulin (*Areg*) gene expression in null skin explants (32). However, such supplementation did not markedly increase EGF and EGFR expression in the submandibular salivary glands of *Tabby* mice during development (33). It is therefore tenable that Eda-induced downstream signaling stimulation of epithelial proliferation may be tissue specific. In our study, we found the EGF-EGFR signaling pathway was down-regulated in the corneal epithelium of *Tabby* mice, whereas *EGFR* gene and protein expression were both up-regulated in HCE cells exposed to Eda, and in Eda-treated *Tabby* mice corneal epithelium, indicating EGFR signaling activation may occur downstream from Eda-induced Edar signaling activation, or the two signaling pathways may interact with one another through cross-talk. Further study is warranted to elucidate how these two signaling pathways interact with one another to mediate Eda-induced increases in ocular surface epithelial proliferation.

In summary, our results show that Eda, one of the major mediators of ectodermal organ morphogenesis, is highly expressed and secreted by meibomian glands and increases corneal epithelial cell proliferation. Because corneal and conjunctival epithelial cells also produce low levels of Eda, we could not rule out the possibility that Eda expressed from basal cells play a role in the proliferation of the epithelium. However, the majority of Eda protein was produced by the meibomian gland, we thus propose that MGD-related ocular surface abnormalities may be attributable to not only declines in lipid secretory activity, but also reduction of functional proteins such as Eda. Our study provides novel insight showing the importance of meibomian gland function for maintaining ocular surface homeostasis. This realization suggests that tear film supplementation with recombinant Eda protein may provide a novel approach to improve treatment of MGD-related ocular surface diseases and other corneal epithelial related diseases in a clinical setting.

Experimental procedures

Antibodies

The following antibodies were used in this study: anti-Eda (SC-18925, Santa Cruz), anti-Edar (ab137021, Abcam), anti-Ki67 (ab66155, Abcam), anti-EGFR (ab52894, Abcam), anti-phospho-ERK1/2 (4695S, Cell Signaling Technology), anti-phospho-EGFR (ab40815, Abcam), and anti- β -actin (A3854, Sigma). The secondary antibodies were Alexa Fluor 488-conjugated donkey anti-rabbit (A21206, Invitrogen) or goat IgG antibody (A11055, Invitrogen), HRP-conjugated rabbit anti-goat (172-1034, Bio-Rad) or goat anti-rabbit antibodies (170-1019, Bio-Rad).

Animals

Eda mutant *Tabby* mice ($C57BL/6J$ - A^{W-J} - $Eda^{Ta-6J/J}$) and wild-type $C57BL/6J$ mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME), were housed and bred at the Experimental Animal Center of Xiamen University. All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement

Ectodysplasin A promotes epithelial cell proliferation

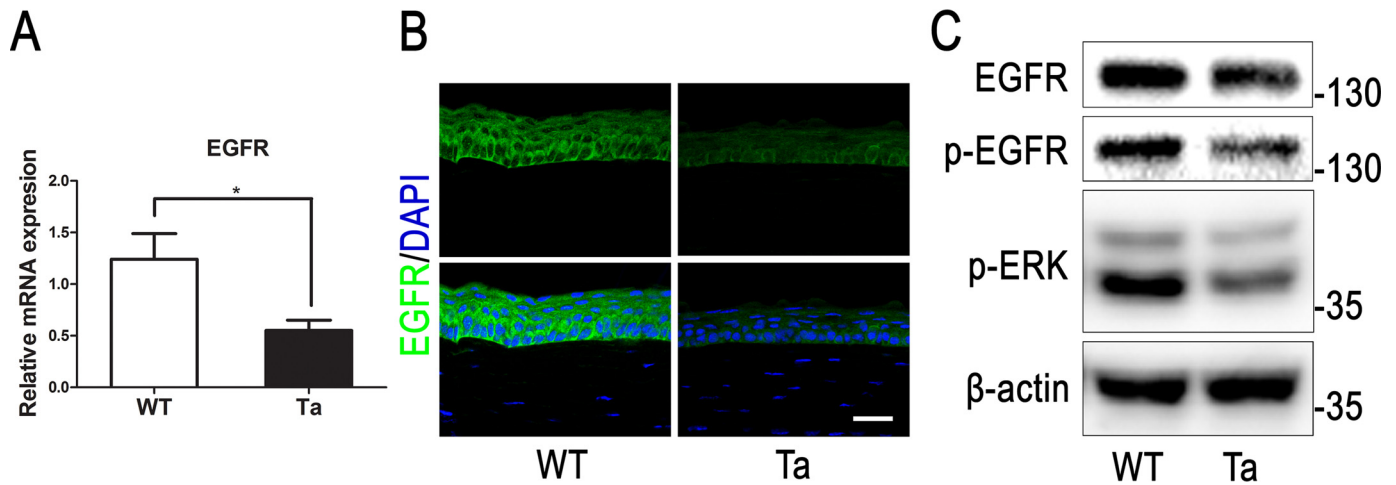


Figure 8. Down-regulation of EGF-EGFR signaling in *Tabby* mice corneal epithelium. *A*, the qRT-PCR result showed *EGFR* mRNA expression decreased in *Tabby* mice corneal epithelium when compared with wild-type littermates ($n = 4$, $*$, $p < 0.05$). *B*, immunofluorescent staining showed EGFR expression on corneal epithelium was much weaker in *Tabby* mice compared with that in wild-type mice, especially in the basal layer. Scale bar represents 50 μm . *C*, Western blot analysis showed both EGFR, p-EGFR and p-ERK levels underwent down-regulation in *Tabby* mice.

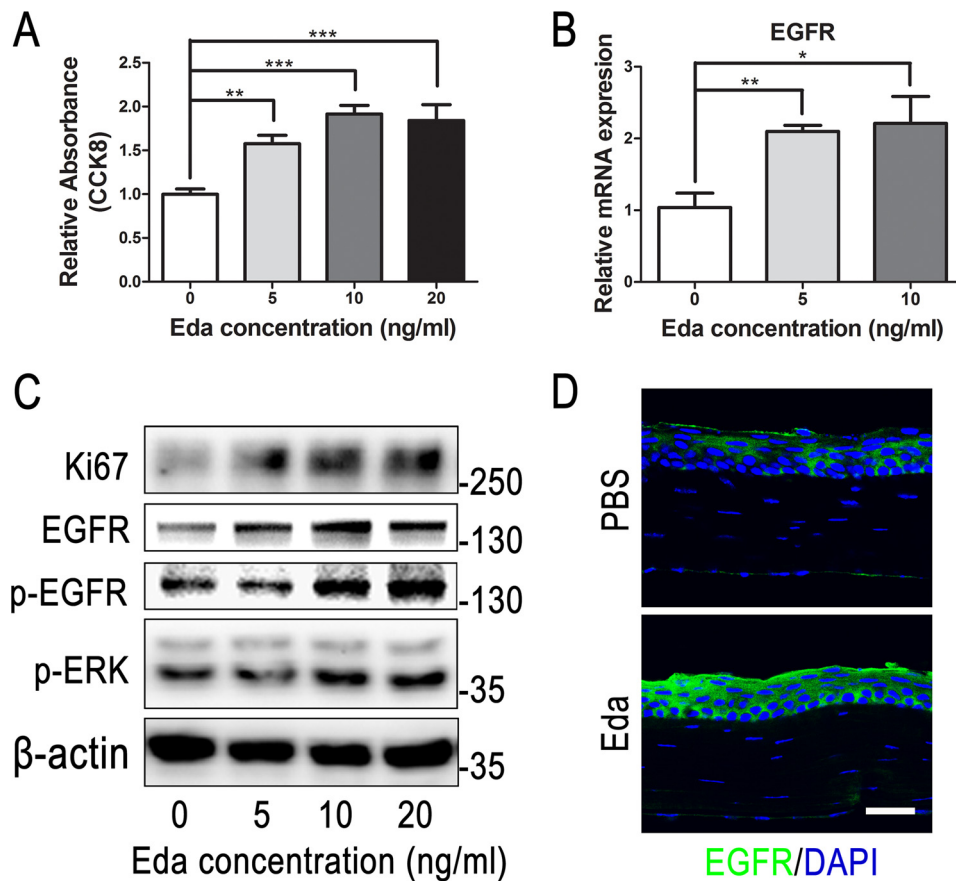


Figure 9. Eda activated EGF-EGFR signaling pathway in HCE cells. *A*, HCE cells were cultured with different concentrations of Eda (0, 5, 10, and 20 ng/ml). CCK8 assay results showed 5 and 10 ng/ml of Eda-enhanced cell proliferation, and reached a plateau at 10 ng/ml ($n = 4$, $**$, $p < 0.01$; $***$, $p < 0.001$). *B*, qRT-PCR analysis showed that *EGFR* mRNA was up-regulated in HCE cells after treatment with 5 and 10 ng/ml of Eda for 4 h ($n = 4$, $*$, $p < 0.05$; $**$, $p < 0.01$). *C*, Western blot analysis showed that Eda treatment up-regulated the expression of Ki67, EGFR, p-EGFR, and p-ERK in a dose-dependent manner. *D*, immunofluorescent staining showed EGFR expression was obviously increased in the corneal epithelium of *Tabby* mice after Eda treatment for 3 weeks when compared with *Tabby* mice treated with PBS. Scale bar represents 50 μm .

for the use of Animals in Ophthalmic and Vision Research and approved by the Animal Ethical Committee of the Eye Institute of Xiamen University (protocol number 2012002). All experiments were performed in accordance with the tenets set forth in the Declaration of Helsinki.

Immunostaining

Mouse ocular tissue cryostat sections (6 μm in thickness) and corneal whole-mount tissues were fixed in 4% paraformaldehyde for 20 min. The samples were washed 3 times with PBS, followed by incubation in 0.2% Triton X-100 for 20 min. After

washing each sample three times with PBS for 5 min and preincubating with 2% bovine serum albumin (BSA) for 1 h at room temperature, sections or corneal tissues were incubated with anti-Eda (1:50), Edar (1:100), Ki67 (1:100), and EGFR (1:100) primary antibodies at 4 °C overnight. After washing each sample with PBS for 10 min, they were incubated with Alexa Fluor 488-conjugated donkey anti-rabbit or goat IgG secondary antibody for 1 h at room temperature. After three additional washes for 10 min each with PBS, the samples were counterstained with DAPI (H-1200, Vector) and then mounted for analysis under the confocal laser scanning microscope (Fluoview 1000, Olympus, Japan). For the whole mount staining of Ki67, the confocal images of central basal corneal epithelial cells were taken, total cell numbers and Ki67 positive cell numbers were counted in three images, the percentages of Ki67-positive cells were calculated and summarized.

Western blot analysis

The mouse cornea, conjunctiva, lacrimal gland, meibomian gland tissue, or HCE cells were extracted with a cold lysis buffer comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitor mixtures. The protein concentration was quantified using BCA assay (23225, Thermo Scientific). The tissue or cell lysate with equal amounts of proteins were subjected to electrophoresis on 6 or 10% SDS-PAGE and then electrophoretically transferred to a PVDF membrane. After blocking in 1% BSA for 1 h, the membranes were incubated with primary antibodies Eda (1:500), Edar (1:1000), Ki67 (1:1000), EGFR (1:1000), phosphor-EGFR (1:1000; Abcam), phosphor-ERK1/2 (1:1000), and β -actin (1:8000) overnight at 4 °C. After three washes with Tris-buffered saline containing 0.05% Tween 20 for 10 min each, the membranes were incubated with HRP-conjugated rabbit anti-goat or goat anti-rabbit IgG for 1 h. Immune complexes were detected by enhanced chemiluminescence reagents and recorded with a Bio-Rad imaging system (ChemiDoc XRS, Bio-Rad). The band intensities were normalized to the corresponding value of β -actin expression.

Human tear and serum collection

Tear samples were collected from 16 healthy volunteers (12 females and 4 males, average age: 26.56 ± 1.87 years) and 4 patients (1 females and 3 males, average age: 18.50 ± 7.37 years) who were diagnosed with MGD based on the criteria of the MGD workshop (34). Informed consent was obtained from all the subjects and the study was approved by the Ethical Committee of the Eye Institute of Xiamen University (protocol number 2012003). Tears were harvested with sterile capillary tubes under the slit-lamp microscope without local anesthesia. During the collection, the lower eyelid was gently pulled down and the tip of the open capillary tube was placed in contact with the tear meniscus without irritating the conjunctiva. A minimum of 2 μ l of tears was collected from each subject. Human serum from 21 healthy volunteers (10 females and 11 males, average age: 32.60 ± 2.36 years) was harvested from the blood by centrifugation. All the tear and serum samples were stored at -80 °C before the measurements.

ELISA

The Eda ELISA kit (DY922, R&D Systems) measured Eda protein concentration by following the manufacturer's protocol in the human tears and serum. Tear samples were, respectively, adequately diluted using PBS. The dilution ratio was recorded. The optical absorbance was measured at 450 nm with a Bio-Tek Elx800 microplate reader (Bio-Tek Instruments, Winooski, VT). The Eda concentration was calculated according to the standard curve.

Hematoxylin and eosin staining

The eyeballs harvested from mice after sacrifice were immediately fixed in 4% paraformaldehyde overnight, or fixed after organ culture for 36 h. The fixed samples were embedded in OCT for performing the histological analysis. Six- μ m thick sections were stained with hematoxylin and eosin and examined under a light microscope.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was isolated from the samples using TRIzol reagent (15596-018, Invitrogen) and was reverse transcribed to cDNA by the ExScript RT Reagent kit (DRR035A, Takara) following the manufacturer's protocol. qRT-PCR was performed with a StepOne Real-Time detection system (Applied Biosystems, Alameda, CA) using an SYBR Premix Ex Taq Kit (RR420A, Takara). The amplification program included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 57 °C for 30 s, and 75 °C for 10 s. Melting curve analysis was conducted at once by raising the temperature from 65 to 95 °C. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. The human and mouse gene primers were designed using the Primer 3 system and their sequences are showed as follows: mouse β -actin, 5-cctaagcc-aaccgtgaaaag-3, 5-aggcatacaggacagcacag-3; mouse *Ki67*, 5-agctcctgctgttggag-3, 5-tcagcctcacagg ctcatct-3; mouse *EGFR*, 5-gagtgactgtctgtctgcc-3, 5-tgtcaccacgtagtttcggg-3; human β -actin, 5-tgacgtggacatccgcaaag-3, 5-ctggaaggtggacagcgagg-3; human *EGFR*, 5-ttgccgcaaagtgtgtaacg-3, 5-accctgtgatttctttacggg-3. The results of the relative qRT-PCR were analyzed by the comparative threshold cycle (C_T) method and normalized to β -actin expression as the reference gene.

EdU labeling and staining

Eight-week-old *Tabby* mice or wild-type mice were intraperitoneally injected twice with EdU at a dose of 25 mg/kg of body weight on consecutive days. The mice were sacrificed 24 h after the second injection, and the eyeballs were harvested and embedded in OCT for frozen section. EdU incorporation into DNA was detected using the Click-iT EdU Alexa Fluor Imaging Kit (C10632, Life Technologies) according to the manufacturer's protocol. The number of EdU-positive cells in the corneal epithelium of sections from three individual *Tabby* mice or wild-type mice were counted.

Corneal epithelial debridement wound

Experimental mice were anesthetized by intraperitoneal injection of 0.2% chloral hydrate. The central corneal epithelium

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lium was demarcated with either a 2- or 2.5-mm trephine and removed using an Algerbrush II rotating burr (Alger Equipment Co., Inc., Lago Vista, TX) under the operating microscope. The denuded epithelial area was stained with 0.1% sterile sodium fluorescein solution in PBS immediately after the wound and at 12, 24, and 36 h after the wound. The surface areas of the wound were quantitated using ImageJ software based on fluorescein staining images. The epithelial wound healing rate was calculated as the percentage of wound closure at different time points. In some studies, the animals were sacrificed after corneal epithelial debridement wound, the eyeballs were nucleated and cultured in 24-well plate with the medium of DMEM + 2% FBS in the absence or presence of 20 ng/ml of mouse recombinant Eda protein or 10 ng/ml of mouse recombinant EGF protein for different durations.

Corneal epithelial cell culture

The human corneal epithelial (HCE) cell line was obtained from RIKEN BioSource Center (Tokyo, Japan) and cultured in DMEM/F-12 (C11330500BT, Gibco) supplemented with 6% fetal bovine serum (900-108, Gemini), 25 mM insulin (I3536, Sigma), and 10 ng/ml of human recombinant epidermal growth factor (AF-100-15, Peprotech). In some experiments, different concentrations of human recombinant Eda protein (3944-ED, R&D) were added to the culture medium when the cells reached about 70% confluence. After 6 or 24 h culture, the culture medium was removed and the cells were washed once with PBS. After that, the cells were used for mRNA or protein collection.

Cell migration assay

HCE cells were seeded into 12-well plates and grown until reaching confluence. The cell layers were then scratched into a cross-shaped wound using a 200- μ l pipette tip. After washing with PBS for three times, the culture medium was replaced with DMEM/F-12 basal medium containing 5, 10, or 20 ng/ml of Eda protein. Control dishes were similarly scratched and cultured in medium without addition of Eda protein. After a 20-h culture, each well was photographed and the extent of healing was measured by ImageJ software.

Cell proliferation assay

To evaluate proliferative capacity of the HCE cells treated with Eda protein, they were seeded at 8000 cells per well in 96-well culture plates. Eda at different concentrations (5, 10, and 20 ng/ml) was added into the complete culture medium after cell attachment. Untreated HCE cells exposed to the same volume of complete culture medium served as a normal control. After 24 h culture, the medium was replaced by CCK8 constituted medium (C0038, Beyotime Biotechnology) and incubated for 4 h at 37 °C in the dark. The optical density of the collected medium was measured at 570 nm with a Bio-Tek ELX800 microplate reader.

Statistical analysis

Statistical analysis was performed with SPSS 16.0.0 (SPSS, Chicago, IL). All data were expressed as mean \pm S.D. Comparisons between groups were performed by independent

Student's *t* test whereby $p < 0.05$ was considered statistically significant.

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