Ectodysplasin A protein promotes corneal epithelial cell proliferation

Received for publication, June 23, 2017. Published, Papers in Press, June 27, 2017, DOI 10.1074/jbc.M117.803809

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Edited by Eric R. Fearon

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) is a type II transmembrane protein belonging to the tumor necrosis factor (TNF) superfamily. It 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 (LTβ) pathways (7–9) mediate hair as well as other ectodermal tissue development rather than the well-documented nuclear factor κB (NF-κB) 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 developmental 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 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...
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. 1A). Western blot analysis confirmed differences in corneal, conjunctival, lacrimal gland, and meibomian gland Eda and Edar expression patterns (Fig. 1B). 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 0.57 ± 0.07 ng/ml in tears from MGD patients (*, p < 0.05). Scale bars represent 50 μm.

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. 2A), 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. 2B).

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
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 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 μl 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 corneas 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
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 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.

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.

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-
Ectodysplasin A promotes epithelial cell proliferation

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...
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 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 epithilium 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.
In previous studies, it was shown that Eda promoted epithelial cell proliferation through the NF-κB 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/6-J-Eda^W-1/Eda^Ta-6J/J) and wild-type C57BL/6 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.
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 pre-incubating 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 electrophotorectively 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 µl 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-µm 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'-cttagggcaccgtgaaag-3', 5'-aggcatacagggacagcacag-3'; mouse Ki67, 5'-acgtcctgcctgtttggaag-3', 5'-tcagcctcacaggtctcatct-3'; mouse EGFR, 5'-gagtgactgtctggtctgcc-3', 5'-tgcaccaagtgctggtgcg-3'; human β-actin, 5'-tgacgtggacatccgcaaag-3', 5'-ctggaaggtggacctcagag-3'; human EGFR, 5'-ttggcaaaagtgtgtaacg-3', 5'-accctgtagctttcctagg-3'. The results of the relative qRT-PCR were analyzed by the comparative threshold cycle (Ct) 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 intra-peritoneally 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 epithe-
Ectodysplasin A promotes epithelial cell proliferation

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 ± 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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Ectodysplasin A promotes epithelial cell proliferation

J. Biol. Chem. (2017) 292(32) 13391–13401