ADAM17 Transactivates EGFR Signaling during Embryonic Eyelid Closure

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Purpose. During mammalian embryonic eyelid closure ADAM17 has been proposed to play a role as a transactivator of epidermal growth factor receptor (EGFR) signaling by shedding membrane bound EGFR ligands. However, ADAM17 also sheds numerous other ligands, thus implicating ADAM17 in additional molecular pathways. The goal of this study was to experimentally establish the role of ADAM17 and determine ADAM17-mediated pathways essential for the embryonic eyelid closure.

Methods. Wild-type (WT) and *woe* mice, carrying a hypomorphic mutation in *Adam17*, were evaluated using H&E and scanning electron microscopy. Expressions of ADAM17, EGFR, and the phosphorylated form EGFR-P were evaluated using immunohistochemistry. BrdU and TUNEL assays were used to evaluate cell proliferation and apoptosis, respectively. In vitro scratch assays of primary cultures were used to evaluate cell migration. Clinical and histologic analyses established if the hypermorphic $Egfr^{Dsk5}$ allele can rescue the *woe* embryonic eyelid closure.

Results. *woe* mice exhibited a failure to develop the leading edge of the eyelid and consequently failure of the embryonic eyelid closure. Expression of ADAM17 was identified in the eyelid epithelium in the cells of the leading edge. ADAM17 is essential for epithelial cell migration, but does not play a role in proliferation and apoptosis. EGFR was expressed in both WT and *woe* eyelid epithelium, but the phosphorylated EGFR-P form was detected only in WT. The $Egfr^{Dsk5}$ allele rescued *woe* eyelid closure defects, but also rescued *woe* anterior segment defects and the absence of meibomian glands.

Conclusions. We provide in vivo genetic evidence that the role of ADAM17 during embryonic eyelid closure is to transactivate EGFR signaling. (*Invest Ophthalmol Vis Sci.* 2013;54:132–140) DOI:10.1167/iovs.12-11130

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Supported by the National Eye Institute/National Institutes of Health Grant EY18872 (DJS).

Submitted for publication October 12, 2012; revised November 14, 2012; accepted November 28, 2012.

Disclosure: **E.L. Hassemer** None; **B. Endres**, None; **J.A. Toonen**, None; **A. Ronchetti**, None; **R. Dubielzig**, None; **D.J. Sidjanin**, None

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ADAM17 belongs to a disintegrin and metalloproteases (ADAMs) family of type I transmembrane Zn^{2+}-dependent proteases that play a role in the proteolytic ectodomain release or "shedding" of membrane-tethered precursors.[1] The role of ADAM17 has been shown to be a sheddase of various proteins, including cytokines and cytokine receptors, growth factors, and adhesion molecules. In fact, as many as 76 proteins have been shown to be substrates for ADAM17 shedding activity.[2] With such functional diversity of substrates, ADAM17 has been implicated in many developmental and disease mechanisms.[3] Analysis of ADAM17 function in vivo showed that most $Adam17^{-/-}$ mice die between embryonic day (E) 17.5 and birth,[4,5] but some $Adam17^{-/-}$ mice survive until weaning.[4] Phenotypically, $Adam17^{-/-}$ mice exhibit heart valve abnormalities; eyelids open at birth (EOB); and defects in maturation of epithelia from lung, skin, and mammary glands, as well as waviness of the fur and vibrissae.[4,5] Recently, an *Adam17* c.C794T substitution was identified in a mouse termed *waved with open eyelids* (*woe*) that resulted in aberrant *Adam17* splicing and severely reduced ADAM17 sheddase activity.[6] Although severely reduced, the ADAM17 sheddase activity was sufficient to reduce the severity of cardiac valve phenotypes associated with the absence of ADAM17 function, thus allowing *woe* mice to survive into adulthood.[4–6] In addition to mild cardiac abnormalities, *woe* mice exhibit EOB and wavy fur phenotypes[6] similar to those of $Adam17^{-/-}$ mice.[4,5] The survival of *woe* mice into adulthood facilitated the identification of previously unknown phenotypes associated with ADAM17 deficiency such as ocular anterior segment abnormalities and the absence of meibomian glands.[6]

The presence of the EOB phenotype in *woe* and $Adam17^{-/-}$ mice provided convincing evidence that ADAM17 is critical for proper embryonic eyelid closure; however, the precise role of ADAM17 during this process has not yet been established. Embryonic eyelid closure is common to all mammals where, following formation, eyelids migrate across the cornea, fuse together, and ultimately reopen.[7] Closed embryonic eyelids serve as a protective barrier by preventing premature exposure of the developing ocular structures to the environment. Both epidermal growth factor receptor (EGFR) signaling and transforming growth factor beta (TGFβ)/activin-mediated MAP3K1/JNK/cJUN signaling pathways have emerged to be essential in facilitating eyelid epithelial cell migration and proper embryonic eyelid closure. Failure of the embryonic eyelid closure is present in mice that carry mutations resulting in abolished or severely reduced EGFR signaling.[8–17] Similarly, mice with mutations in genes encoding members of the TGFβ/activin-mediated MAP3K1/JNK/cJUN signaling also exhibit EOB phenotypes.[18–22] Although both signaling pathways are involved in epithelial cell migration, it remains unclear how these two signaling pathways are coordinated. ADAM17 has been proposed as a sheddase of EGFR ligands during embryonic eyelid closure and, consequently, a transactivator of EGFR signaling.[9] However, in cancer cells, ADAM17 has been implicated in the regulation of TGFβ[23]

Therefore, it is possible that during embryonic eyelid closure, in addition to shedding EGFR ligands and transactivation of EGFR signaling, ADAM17 may be also regulating TGFβ and, consequently, transactivating TGFβ/activin-mediated MAP3K1/JNK/cJUN signaling. Therefore, ADAM17 may play a critical role in coordinating these two molecular pathways.

As a part of this study, our goal was to experimentally clarify the role of ADAM17 during embryonic eyelid closure. In addition, our goal was to establish the molecular pathways and mechanisms governed by ADAM17 during this process. Our results showed that ADAM17 is indispensable for the formation of the leading edge at the tips of developing eyelids just prior to the initiation of eyelid closure. In addition, we demonstrate that ADAM17 is essential for cell migration, but not for proliferation or apoptosis. Finally, we provide genetic evidence that ADAM17 regulates embryonic eyelid closure through direct transactivation of the EGFR signaling pathway.

Materials and Methods

Mice, Clinical Evaluation, and PCR Genotyping

C57BL/6J and *woe* mice were maintained as previously described.[6] *Dsk5* mice were provided by David Threadgill, PhD, from the University of North Carolina. All mice were used with strict adherence to the guidelines set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For clinical evaluation, mouse eyes were evaluated as previously described.[24,25] The *Egfr*^{Dsk5} and *Adam17*^{woe} alleles were genotyped as previously described.[6,26]

Histology, Immunohistochemistry, and Electron Microscopy

Embryonic day 0.5 (E0.5) was defined as the morning of the day that a vaginal plug was first observed. Embryonic heads were collected, fixed, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin (H&E) as previously described.[6] Antigen retrieval was performed by boiling samples in a microwave oven for 10 minutes in sodium citrate (10 mM, pH 6.0). Endogenous peroxidase activity was quenched using 1% H_2O_2 in 100% methanol (40 minutes, room temperature) followed by 3 × 5-minute washes in PBS and 2 × 10-minute washes in 0.3% Triton/PBS. For immunostaining, a commercial immunodetection kit (Vector Mouse on Mouse [M.O.M.] Immunodetection Kit; Vector Laboratories, Burlingame, CA) was used with anti-ADAM17 (Abcam, Cambridge, MA), anti-EGFR (Cell Signaling Technology [CST], Beverly, MA), and anti-EGFR-P (CST) antibodies at 4°C O/N following manufacturer's protocol. A commercial ready-to-use reagent (VECTASTAIN Universal Elite ABC Kit; Vector Laboratories) was used following the manufacturer's protocol. Tyramide-Cy3 was then added to the horseradish peroxidase using a tyramide signal amplification (TSA)-cynanine 3 kit (NEL 744; PerkinElmer, Inc., Waltham, MA) at a dilution of 1:50 for 3.5 minutes, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies, Carlsbad, CA). Slides were then mounted and photographed with a digital camera (Nikon DS-Fi1 Camera; Nikon Instruments Inc., Melville, NY) on a digital microscope (Nikon Eclipse 80i; Nikon Instruments Inc.). Tissues for electron microscopy were fixed in a 2% glutaraldehyde solution and then processed for scanning electron microscopy as previously described[27] and photographed with a scanning electron microscope (XL30; FEI/Philips, Tustin, CA).

Isolation and Culture of Keratinocytes, Dermis Fibroblasts, and Mouse Embryonic Fibroblasts

Primary mouse embryonic fibroblasts (mEFs) were generated and cultured as described previously.[6] Isolation and culture of keratinocytes and fibroblasts were performed following a previously established protocol.[28] The keratinocytes were cultured in K-SFM medium supplemented with bovine pituitary extract, and 10 ng/mL EGF, 1.3 mM calcium, or 0.05 mM calcium, and 1% penicillin–streptomycin (Life Technologies). Dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin (Life Technologies).

BrdU Incorporation and TUNEL Assay

Mice were injected intraperitoneally at E15.5 with 100 μg/g body weight of 5-bromo-2′-deoxyuridine (BrdU) and euthanized 2 hours later. Embryo heads were collected, fixed in 4% paraformaldehyde (PFA), paraffin-embedded, and sectioned using standard protocols. Immunostaining for BrdU was carried out using a commercial staining kit (Zymed BrdU Kit; Life Technologies) following the manufacturer's protocol. The numbers of BrdU-positive cells in the eyelids were counted as previously described.[29] The means and SEM were calculated and deemed significant if $P < 0.05$ as determined by a Student's *t*-test. To measure the proliferation rate in cell culture, WT and *woe* keratinocytes or WT and *woe* fibroblasts were grown to 80% confluency and incubated with 50 μg/mL BrdU at 37°C in 5% CO_2 for 4.5 hours, fixed in 70% cold methanol, and immunostained using a commercial kit (Zymed BrdU Kit; Life Technologies) following the manufacturer's protocol. The percentage of BrdU-positive cells as compared with total cell number was then calculated and deemed significant if $P < 0.05$, as determined by a Student's *t*-test.

A TUNEL assay was done with a commercial detection kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit; EMD Millipore Corp., Billerica, MA) following the manufacturer's protocol. Following TUNEL assay slides were counterstained with hematoxylin. TUNEL-positive cells were counted. The means and SEM were calculated and considered significant if $P < 0.05$, as determined by Student's *t*-test.

In Vitro Scratch Assay

WT and *woe* mEFs or keratinocytes were seeded onto 12-well culture dishes, scratched with a 200 μL pipette tip, and cultured in medium containing no supplements, or containing medium + TGFα (10 ng/mL), or containing medium + $TGF\beta_1$ (10 ng/mL). Images were taken immediately after scratching and 48 hours later. The number of cells that migrated into the gap was counted in three random fields at 50 different positions.[28,30] Differences were deemed significant if $P < 0.05$, as determined by an unpaired *t*-test. For F-actin immunostaining, cells were rinsed twice with PBS, fixed with fresh methanol-free 3.7% PFA for 10 minutes, permeabilized with 0.1% Triton-X for 5 minutes, blocked with 1% bovine serum albumin for 30 minutes, and incubated with phalloidin conjugated to Alexa Fluor 488 (Life Technologies). DNA was counterstained with DAPI (Life Technologies), and photographed with a digital camera on a digital microscope (Nikon DS-Fi1 and Nikon Eclipse 80i, respectively; Nikon Instruments Inc.).

Results

ADAM17 Is Essential for the Formation of the Leading Edge during Embryonic Eyelid Closure

As the initial step in determining the role of ADAM17 during embryonic eyelid closure, we set out to morphologically examine the embryonic eyelid closure defect in *woe* mice. At E13.5, the primitive eyelids formed and no obvious morphologic differences were noted between WT and *woe* mice (Figs. 1A, 1E). By E15.5, the leading edges formed and started migrating toward each other in WT mice (Fig. 1B); in contrast, the leading edge failed to form in *woe* mice (Fig. 1F). In WT mice, the leading edges met and formed the eyelid closure by E16.5 (Fig. 1C), whereas in *woe* mice the eyelids remained wide open, indicating failure of eyelid closure (Fig. 1G).

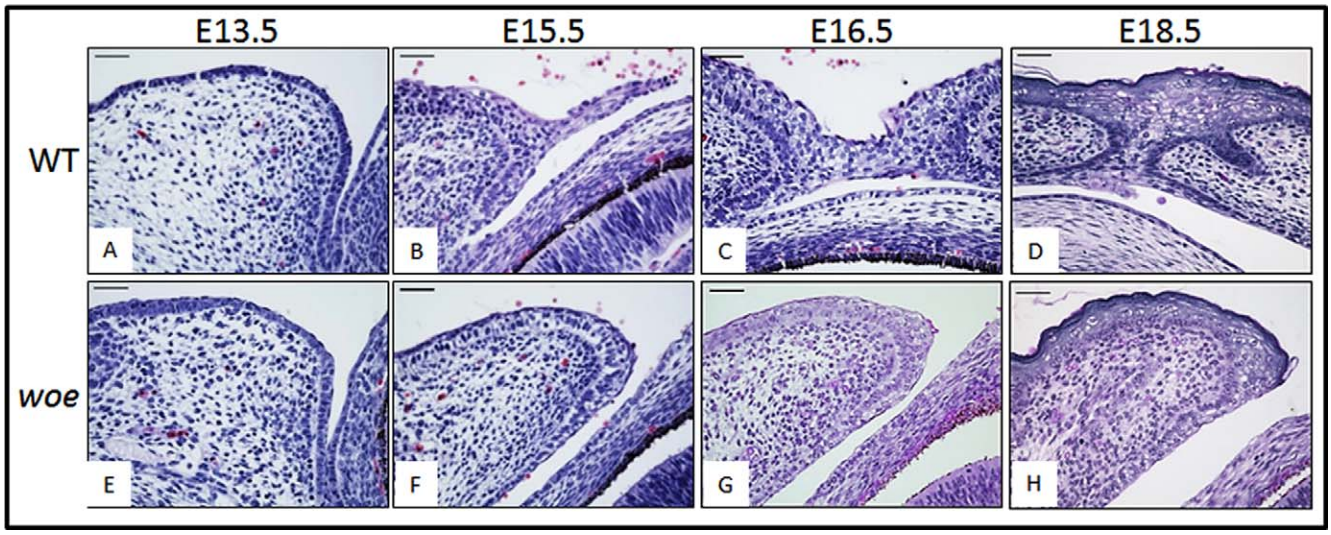

Figure 1. H&E staining of eyelids from WT (*top*) and *woe* (*bottom*) mice. At E13.5, no obvious morphologic differences were observed between WT (**A**) and *woe* (**E**) mice. At E15.5, the leading edge formed in WT mice (**B**), although in *woe* mice the leading edge did not form (**F**). By E16.5, the leading edges met and formed a junction in WT mice (**C**), whereas eyelids in *woe* mice remained open (**G**). Further histologic analysis of *woe* mice at E18.5 (**H**) showed the failure of embryonic eyelid closure, in contrast to WT mice that exhibited completed embryonic eyelid closure (**D**). *Scale bars*, 50 μm.

Embryonic eyelid closure was complete by E18.5 in the WT mice (Fig. 1D), whereas in *woe* mice the eyelids remained open (Fig. 1H). These findings indicated that the initial defect observed in *woe* mice was failure of the formation of the leading edge observed at E15.5. Thus, we sought to further investigate morphologic differences between WT and *woe* eyelids at this developmental time point using scanning electron microscopy (SEM). Eyelids of E15.5 WT mice exhibited fully formed leading edges that were migrating across the cornea (Fig. 2A); higher magnification showed the presence of rounded peridermal cells present at the rims of the leading edges (Fig. 2A′). In contrast, E15.5 *woe* eyelids were fully open, indicating the absence of formation of even rudimentary leading edges (Fig. 2B); higher magnification confirmed this absence of the leading edge, and identified a few rounded peridermal cells at the rims of *woe* eyelids (Fig. 2B′). By E16.5, eyelid closure was completed in WT mice (Fig. 2C), and the eyelid junction was formed with flattened cells present at the eyelid junction (Fig. 2C′). At E16.5, *woe* eyelids remained open and morphologically similar in appearance to the *woe* eyelids observed at E15.5 (Fig. 2D), although more peridermal cells were present at the *woe* eyelid rims at E16.5 (Fig. 2D′) as compared with *woe* eyelid rims at E15.5 (Fig. 2B′).

ADAM17 Expression in the Developing Eyelid

At E13.5, ADAM17 was expressed in the palpebral epithelia, although weak ADAM17 expression was observed in both bulbar epithelial and mesenchymal cells of the eyelid (Fig. 3). At E15.5, ADAM17 was highly expressed in the cells of the leading edge, in addition to the palpebral epithelia of the eyelid (Fig. 3). By E16.5, ADAM17 remained highly expressed in the palpebral epithelia of the eyelid as well as in the cells at the eyelid junction (Fig. 3). By P0.5, ADAM17 was expressed only in the epidermis of the eyelid (Fig. 3).

ADAM17 Does Not Contribute to Cell Proliferation or Cell Death during Embryonic Eyelid Closure

We examined cell proliferation in WT and *woe* eyelids at E15.5 by measuring BrdU incorporation. There was no significant difference between the number of BrdU-positive cells within the epidermis or dermis of WT or *woe* eyelids (Figs. 4A, 4B). To further confirm whether ADAM17 regulates cell proliferation, we isolated primary keratinocytes and dermal fibroblasts from WT and *woe* mice at P0.5 and cultured them in the presence of BrdU. These results showed that there was no significant difference between WT and *woe* in the number of BrdU-positive keratinocytes or dermal fibroblasts (Figs. 4C, 4D). We also examined if ADAM17 may play a role as a regulator of apoptosis during embryonic eyelid closure. However, neither WT nor *woe* E15.5 eyelids exhibited any presence of TUNEL-positive cells (not shown). These results indicate that ADAM17 is not involved in cell proliferation or apoptosis during embryonic eyelid closure.

ADAM17 Is Involved in Cell Migration

As the initial step in evaluation of involvement of ADAM17 during cell migration, we performed an in vitro scratch assay in WT and *woe* mEFs. Previous studies have shown that ADAM17 is expressed in mEFs.[6] Fewer *woe* mEFs migrated into the gap 48 hours after the scratch as compared with WT mEFs (Figs. 5A, 5B). During embryonic eyelid closure, only the epidermal layer is involved in eyelid fusion,[12] with keratinocytes as the major constituent of eyelid epidermis. Therefore, we evaluated whether keratinocytes from *woe* mice exhibited a defect in cell migration similar to the one observed in *woe* mEFs. The results showed that *woe* keratinocytes also exhibited fewer migrating cells following the scratch when compared with keratinocytes from WT mice (Fig. 5B). Next, we investigated the filopodia formation by staining mEFs and keratinocyte cells with phalloidin. In WT mEFs and keratinocytes, filopodia were abundantly present (Fig. 5C). In contrast, fewer filopodia were present in *woe* mEFs and keratinocytes that appeared to be shorter in length (Fig. 5C). TGFα is a well-established substrate of ADAM17[3]; therefore, we hypothesized that exogenous addition of TGFα may rescue the cell migration defect. Exogenous addition of TGFα indeed rescued the migration defect identified in both *woe* mEFs (Fig. 5D) and keratinocytes (not shown). Interestingly, exogenous addition of TGFβ also

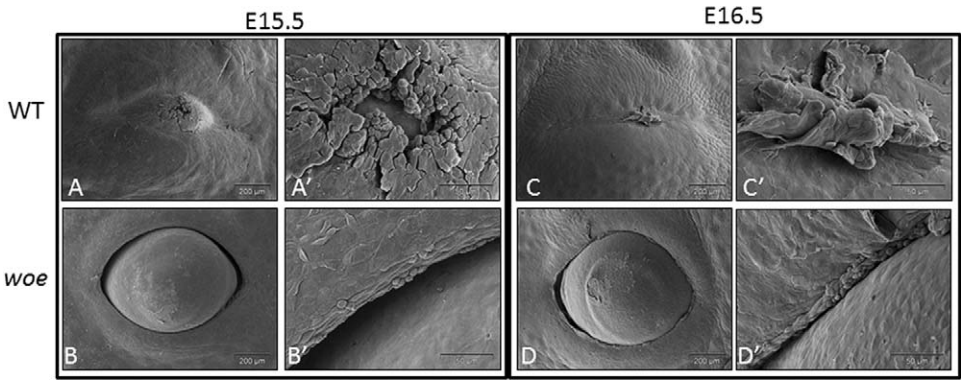

Figure 2. Scanning electron micrographs showing absence of the leading edge formation in *woe* embryos. In E15.5 WT mice (**A**), the formed leading edges were migrating toward each other. The accumulation of rounded peridermal cells was evident at the tips of the leading edges (**A′**). In contrast, *woe* embryos (**B**) exhibited the absence of even rudimentary leading edges, with only a few rounded peridermal cells present at the rims of the eyelids (**B′**). By E16.5 in WT embryos (**C**), the leading edges met, forming the junction (**C′**), whereas in *woe* mice (**D**) the eyelids remained open with a few rounded peridermal cells present at the eyelid margins (**D′**). *Scale bars*: 200 μm (**A**–**D**); 50 μm (**A′**–**D′**).

rescued the cell migration defect in *woe* mEFs (Fig. 5D) and keratinocytes (not shown).

ADAM17 Activates EGFR Signaling during Embryonic Eyelid Closure

We set out to investigate if ADAM17 mediates EGFR signaling during embryonic eyelid closure. Immunohistochemical analysis identified expression of EGFR (Fig. 6A) and phosphorylated EGFR-P (Fig. 6C) in WT palpebral epithelia and in the leading edge cells. In *woe* eyelids EGFR was also expressed in the palpebral epithelia (Fig. 6B), but expression of phosphorylated EGFR-P was severely reduced (Fig. 6D). These findings suggested that at E15.5 just prior to the eyelid closure, ADAM17 facilitates EGFR phosphorylation. Next, we evaluated if ADAM17 mediates only EGFR signaling in vivo or if ADAM17 additionally mediates other signaling pathways essential for embryonic eyelid closure. *Dsk5* is a hypermorphic mutation in the *Egfr* gene, resulting in constitutively active EGFR.[26] *Dsk5* mice exhibit normal eyelid closure and ocular development (not shown). Our results revealed that in *woe* mice (Figs. 7A, 7E), the presence of a single $Egfr^{Dsk5}$ allele resulted in partial eyelid closure, although the eyelid junction did not appear to be fully formed (Fig. 7B); histologic analysis showed that in these mice the eyelid junction was formed at the eye periphery (not shown), but not at the center of the eyelid (Fig. 7F). Mice homozygous for both *woe* and $Egfr^{Dsk5}$ alleles exhibited fully closed eyelids (Fig. 7C), similar in appearance to that of WT mice (Fig. 7D). Histologic analysis confirmed fully formed eyelid junctions in these mice (Fig. 7G), which were morphologically indistinguishable from the eyelid junctions formed in WT mice (Fig. 7H).

$Egfr^{Dsk5}$ Also Rescues Anterior Segment Gland Defects and the Absence of Meibomian Gland Observed in *woe* Mice

Clinically, adult *woe* mice exhibited small eyes with opaque and vascularized corneas (Fig. 8A); histologic analysis identified abnormalities of the corneal epithelium, corneal stroma, the absence of Descement's membrane, and anterior synechiae (Fig. 8E). Clinical evaluation of eyes from *woe* mice carrying a single $Egfr^{Dsk5}$ allele also identified smaller eyes with corneal opacification and neovascularization (Fig. 8B), although the corneal opacity phenotype appeared to be less severe (Fig. 8B) when compared with *woe* mice carrying both WT *Egfr* alleles (Fig. 8A). Histologic analysis of *woe* mice carrying a single $Egfr^{Dsk5}$ allele revealed a hypercellular corneal stroma with vascular spaces distorting the corneal lamellae (Fig. 8F). The corneal epithelium in these mice was intact, although slightly irregular, whereas the corneal endothelium (Fig. 8F) and other ocular structures were within normal limits (not shown). Clinical and histologic analysis of *woe* mice carrying two $Egfr^{Dsk5}$ alleles (Figs. 8C, 8G) identified eyes that were clinically within normal limits and morphologically similar in appearance to those of WT eyes (Figs. 8D, 8H), indicating a rescue of the phenotypes associated with ADAM17 deficiency observed in *woe* mice.

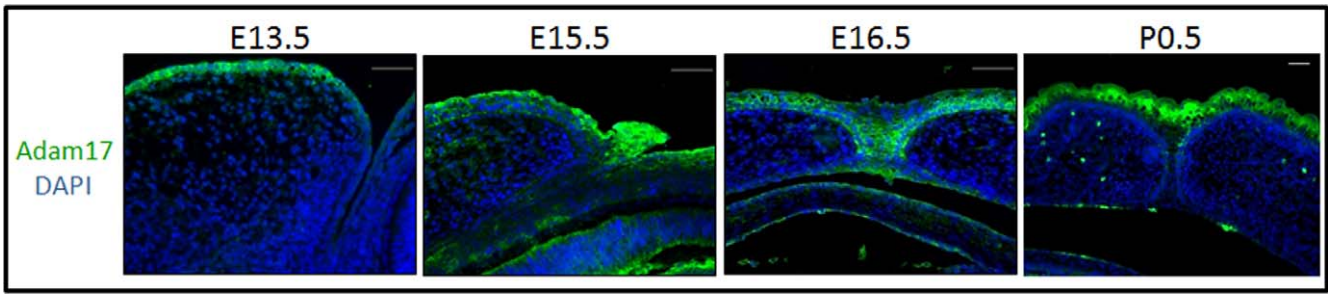

Figure 3. ADAM17 expression in the developing eyelid. In the E13.5 eyelid, ADAM17 (*green*) was expressed in the palpebral epidermis. At E15.5, in addition to the palpebral epidermis, ADAM17 is also highly expressed in the cells of the leading edge. As the eyelids fuse at E16.5, ADAM17 remains expressed in the palpebral epidermis as well as in the cells of the eyelid junctions. By P0.5, ADAM17 is expressed only in the palpebral epidermis. DAPI (*blue*) was used as a nuclear stain. *Scale bars*, 50 μm.

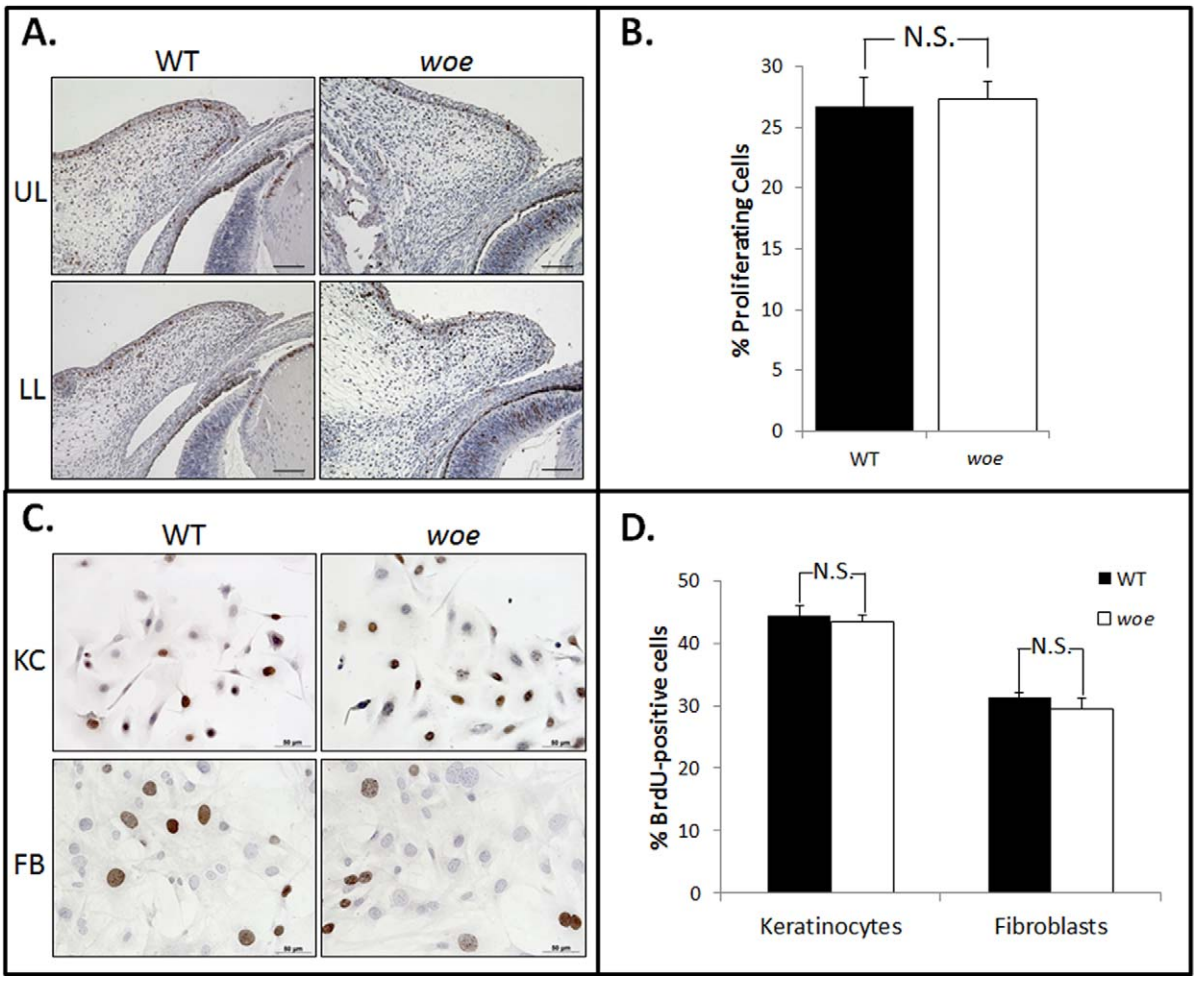

Figure 4. Proliferation in WT and *woe* embryonic eyelids. BrdU incorporation in nuclei of cells from E15.5 WT and *woe* upper lids (UL) and lower lids (LL) (**A**). BrdU-positive (*brown*) cells were observed in both epidermis and dermis. The number of BrdU-positive cells did not differ between WT and *woe* E15.5 eyelids (**B**). The percentage of BrdU-positive cells was calculated from three WT and three *woe* embryos. No difference in proliferation was observed in keratinocytes (KC) or fibroblasts (FB) from WT and *woe* mice (**C**). Quantification of BrdU-positive cells from (**C**) is shown in (**D**). Data represent the mean ± SEM. *Scale bars*, 50 μm. N.S., not significant.

We previously identified the absence of the meibomian gland in *woe* mice.[6] Histologic analysis showed that meibomian glands in mice carrying both *woe* and *Dsk5* alleles did not differ from WT mice (not shown).

Discussion

The main goal of this study was to experimentally establish the role of ADAM17 during embryonic eyelid closure. Even though numerous proteins have been shown to be substrates for ADAM17 sheddase activity, we provide in vivo genetic evidence that the role of ADAM17 during embryonic eyelid closure is to directly transactivate EGFR signaling. EGFR is a cell surface tyrosine kinase receptor that, upon ligand binding, dimerizes and undergoes autophosphorylation events that elicit downstream signaling. Our immunohistochemical analysis demonstrated that prior to the eyelid closure ADAM17 facilitates EGFR phosphorylation. Additionally, the hypermorphic $Egfr^{Dsk5}$ allele fully rescued the *woe* EOB phenotype. $Egfr^{Dsk5}$ mice carry a point mutation in *Egfr* that results in p.Leu863Gln substitution within a three-residue β-strand that helps stabilize the EGFR activation loop and, consequently, results in constitutive EGFR transactivation.[26] Functional analysis showed that the $Egfr^{Dsk5}$ allele rescues phenotypes in *wavy 2* (*wa2*) mice carrying a hypomorphic *Egfr* mutation, further confirming that $Egfr^{Dsk5}$ is a hypermorphic *Egfr* allele.[26] Although $Egfr^{Dsk5}$ mice exhibit defects in skin pigmentation, hair texture, and epidermis,[26] they exhibit normal eyelid closure as well as normal ocular development. The eyelid phenotypes in newborn pups carrying both *woe* and $Egfr^{Dsk5}$ alleles were indistinguishable from eyelid phenotypes in WT mice, thereby establishing the role of ADAM17 during embryonic eyelid closure as a transactivator of EGFR signaling. However, it has been recently shown that EGFR signaling induces cJUN-mediated activation of MAP3K1 during embryonic eyelid closure, which in turn activates the JNK–cJUN pathway.[31] This suggests that ADAM17 may be involved in regulating the MAP3K1-mediated JNK/cJUN pathway, but only indirectly via its transactivation of EGFR signaling.

EGFR ligands include TGFα, amphiregulin, HBEGF, betacellulin, epiregulin, and epigen.[32] Although membrane-bound EGFR ligands can facilitate juxtacrine or autocrine signaling, paracrine EGFR signaling facilitated by ligand shedding is most likely critical for embryonic eyelid closure.[1,33,34] It has been proposed that complete embryonic eyelid closure requires cooperative binding of several EGFR ligands to the EGF receptor.[9] This comes out of the observation that mice

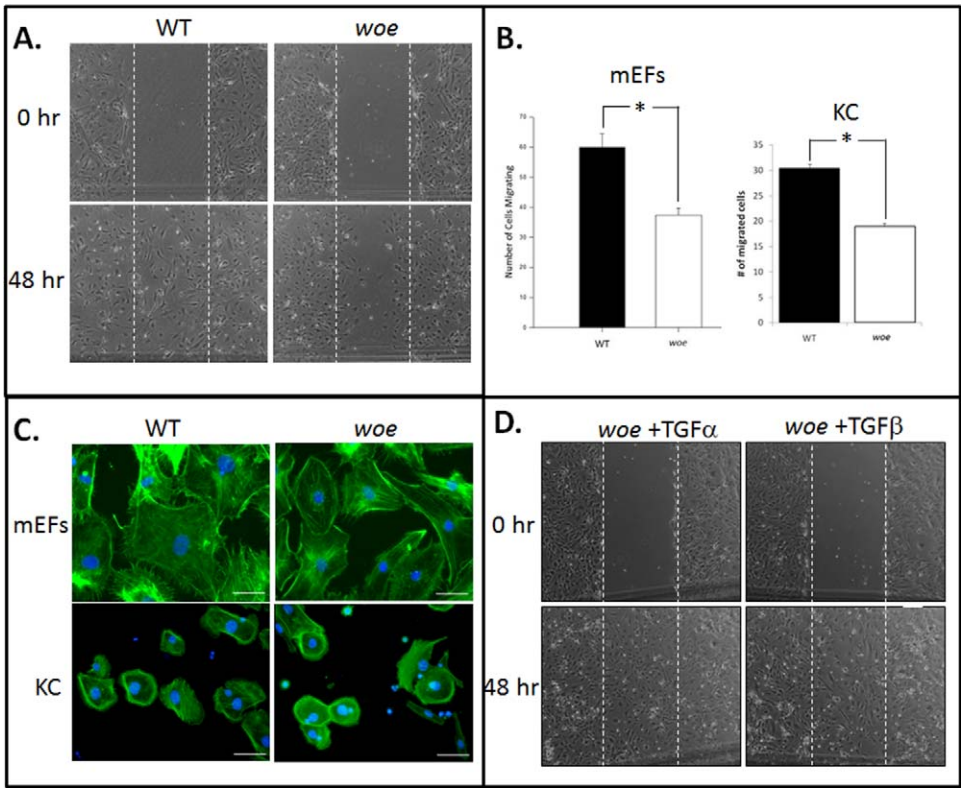

Figure 5. ADAM17 is essential for cell migration. Confluent monolayers of mEFs shown in (**A**) and primary epidermal keratinocytes (not shown) cultured in medium with no growth factors were subjected to in vitro scratch assays. Photographs were taken immediately and 48 hours following the scratch (**A**). Quantification of cells that migrated into the scratch is summarized in (**B**). Both mEFs and keratinocytes were stained with phalloidin conjugated to Alexa Fluor 488 for F-actin (*green*) and DAPI for nuclei (*blue*). Filopodia were abundantly present in WT mEFs and keratinocytes, but not in *woe* mEFs and keratinocytes (**C**). Exogenous addition of TGFα (10 ng/mL) or TGFβ (10 ng/mL) rescued the cell migration defect in *woe* mEFs (**D**) and *woe* keratinocytes (not shown). *Scale bars*, 50 μm. *Asterisks* indicated that the difference in cell migration between WT and *woe* mEFs and keratinocytes was significant ($P < 0.05$).

carrying null alleles of *Egfr* exhibit a fully penetrant EOB phenotype.[15,17,35] In contrast, *Hbegf*$^{-/-}$ and *Tgfα*$^{-/-}$ mice exhibit an incompletely penetrant EOB phenotype.[9] Interestingly, mice carrying both *Hbegf* and *Tgfα* null alleles exhibit the EOB phenotype with increased penetrance as compared with each null genotype independently, although double *Hbegf* and *Tgfα* null allele mice still exhibit an incompletely penetrant EOB phenotype.[9] Mice carrying triple null alleles of *Egf*, amphiregulin, and *Tgfα* also exhibit an incompletely penetrant EOB phenotype.[10] It has been shown that in tissue culture ADAM17 preferentially sheds epiregulin, Tgfα, amphiregulin, and HBEGF,[36,37] whereas ADAM10 preferentially sheds EGF and betacellulin.[37] Taken together it is unclear, in addition to HBEGF and TGFα, which additional EGFR ligands are involved in vivo during embryonic eyelid closure and if ADAM17 is the only sheddase that transactivates EGFR signaling during this process. The fully penetrant EOB phenotype observed in *woe* and *Adam17*$^{-/-}$ implies that ADAM17 is probably the primary sheddase involved in EGFR ligand shedding, although this requires further investigation.

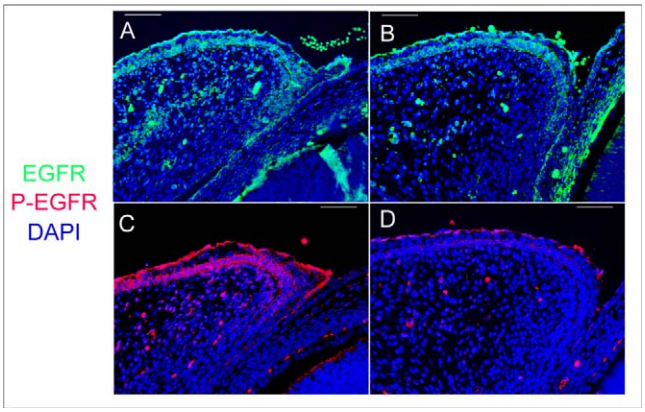

Figure 6. EGFR and EGFR-P expression in E15.5 eyelids. EGFR is expressed in the palpebral epidermis in the eyelids in WT (**A**) and *woe* (**B**) mice; in WT eyelids EGFR is also expressed in the cells of the leading edge (**A**). Expression of phosphorylated EGFR-P is also identified in the palpebral epidermis and in the cells of the leading edge of the WT eyelids (**C**). In contrast, in *woe* epidermis expression of EGFR-P is severely reduced (**D**). *Scale bars*, 50 μm.

Results from our study show that ADAM17 is indispensable for formation of the leading edge and epithelial cell migration. This is consistent with previous findings that the role of EGFR signaling is in the formation of the leading edge and epithelial cell migration.[9] Eyelid closure is a process that shares specific morphogenetic steps with wound healing as well as dorsal closure in *Drosophila* that include the formation of a leading edge of epithelial cells, formation of filopodia and actin stress fibers, cell migration, and reepithelization.[38] The leading edge is a structure composed of epithelial cells that protrudes from the tip of the embryonic eyelids just prior to the cell migration.[7] As the leading edges migrate toward each other, rounded peridermal cells accumulate on the surface of the leading edges.[7] Our results show that ADAM17 is highly

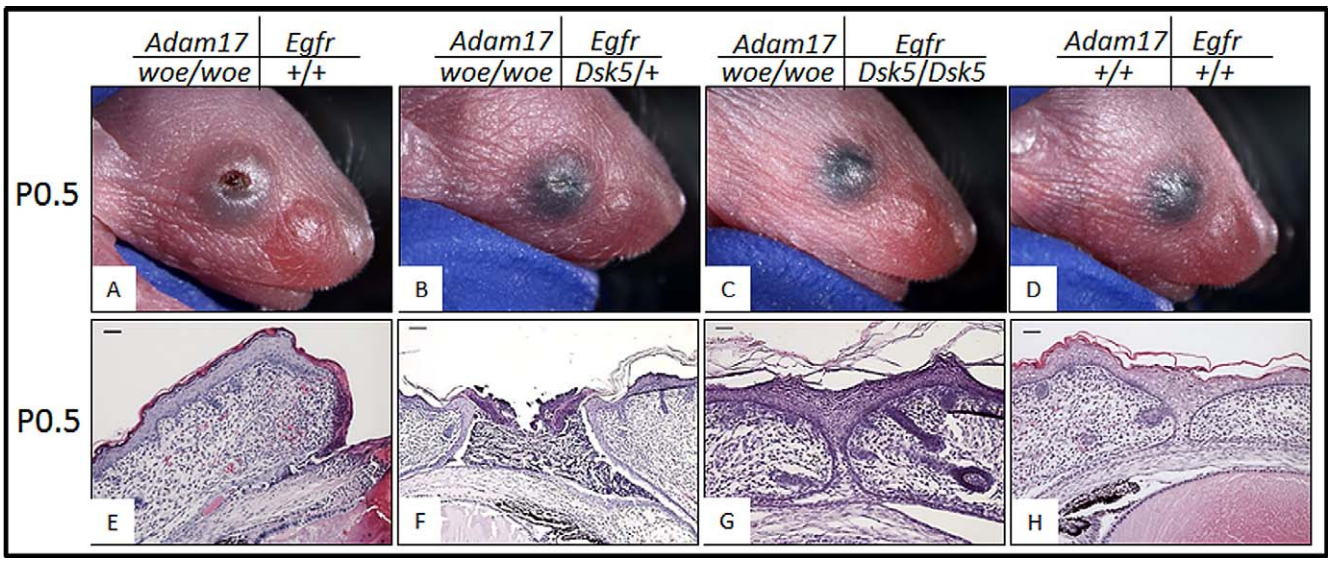

Figure 7. The *Egfr*^{Dsk5} allele rescues the *woe* EOB phenotype. Newborn *woe* mice exhibit the EOB phenotype shown in (**A**); H&E sections of P0.5 *woe* mice confirm the failure of embryonic eyelid closure (**E**). Newborn mice carrying a homozygous *woe* allele and a single *Egfr*^{Dsk5} allele exhibit less severe EOB phenotype (**B**) than that of *woe* mice (**A**). H&E sections of P0.5 mice carrying a homozygote *woe* allele and a single *Egfr*^{Dsk5} allele show the failure of eyelid closure, although the eyelids appear to be closer together with keratinized tissue filling in the interpalpebral aperture (**F**). Newborn mice carrying homozygote *woe* and homozygote *Egfr*^{Dsk5} allele (**C**) exhibit eyelids that are similar in appearance to that of WT mice (**D**). Histologic analysis confirms that eyelid closure in mice carrying homozygote *woe* and homozygote *Egfr*^{Dsk5} alleles, as shown in (**G**), did not differ morphologically from the eyelid junction observed in WT newborn mice (**H**). *Scale bars*, 25 μm.

expressed in the developing eyelid epithelium prior to formation of the leading edge, as well as in the cells of the leading edge. The absence of even rudimentary leading edge structures in *woe* embryonic eyelids resembles the absence of leading edges observed in eyelids from *Fgf10*$^{-/-}$ and *Fgfr2*$^{-/-}$ mice that exhibit EOB phenotypes.[12,39] FGF10 expressed in the developing eyelid mesenchyme, via its receptor FGFR2 expressed in the eyelid epithelium, directly regulates EGFR signaling by regulating expression of TGFα.[12] However, in the skin it has been shown that EGFR-mediated keratinocyte migration is dependent on FGFR2 stimulation of ADAM17.[40] Therefore, we propose that during embryonic eyelid closure, in addition to regulating expression of TGFα, FGF10/FGFR2 signaling may also be directly regulating ADAM17 sheddase activity. Our results also show that ADAM17 facilitates epithelial cell migration as well as filopodia formation, which is consistent with its role as an EGFR transactivator. It should be noted, however, that the exogenous addition of TGFβ rescued the *woe* cell migration defect. Previous studies showed that in vitro activation of either TGFα-mediated EGFR signaling or TGFβ/activin-mediated MAP3K1/JNK/cJUN signaling was sufficient to rescue the cell migration defect in cultured

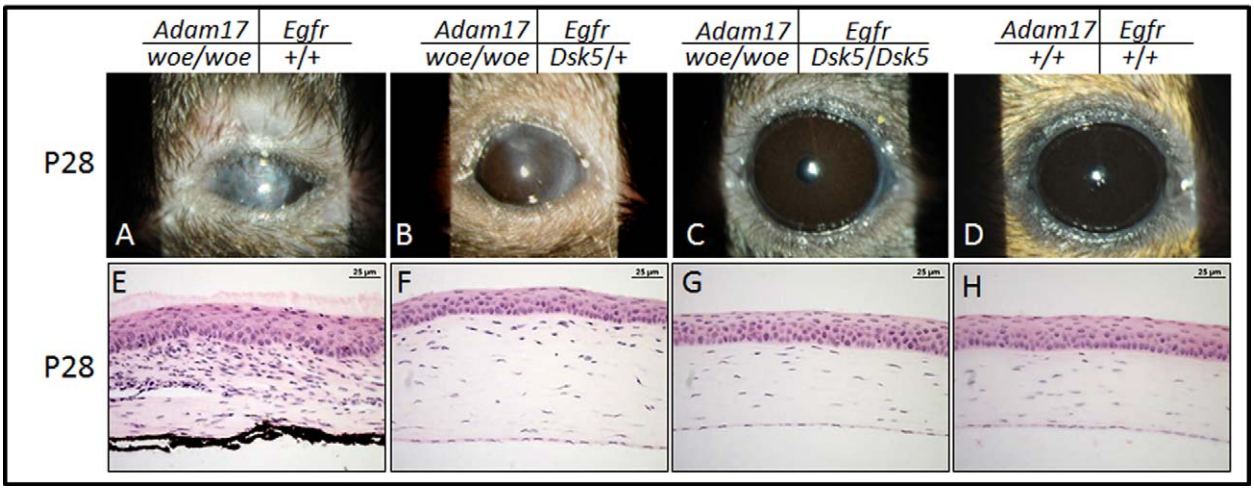

Figure 8. The *Egfr*^{Dsk5} allele rescues the *woe* anterior segment dysgenesis phenotype. Clinical evaluation of *woe* mice at P28 identified severe corneal opacification and corneal neovascularization (**A**). Histologic analysis of *woe* eyes at P28 confirmed severe anterior segment abnormalities, including highly disorganized corneal epithelium, stroma and endothelium, the absence of Descement's membrane, and anterior synechiae as shown in (**E**). Clinical evaluation of homozygote *woe* mice carrying a single *Egfr*^{Dsk5} allele also revealed corneal opacities (**B**) that were less severe than those in *woe* mice (**A**). Histologic analysis of homozygote *woe* mice carrying a single *Egfr*^{Dsk5} allele showed some irregularities of the corneal epithelium, a hypercellular corneal stroma, and abnormalities of the corneal lamellae (**F**). In *woe* mice carrying two *Egfr*^{Dsk5} alleles, clinical (**C**) and histologic (**G**) analyses identified eyes that were clinically (**D**) and histologically (**H**) similar in appearance to that of WT eyes. *Scale bars*, 25 μm.

Map3k1$^{-/-}$ keratinocytes.[18] Our results also show that activation by TGFβ or TGFα in vitro can rescue the cell migration defect in *woe* mEFs and keratinocytes. However, in vivo, the presence of both signaling pathways is required for embryonic eyelid closure and the absence of either signaling pathway results in EOB.

In addition to rescuing the EOB phenotype, the hypermorphic *Egfr*^{Dsk5} allele rescued the anterior segment defects and the absence of meibomian glands previously reported for the *woe* mice.[6] Mice with a mutation in *Tgfa* also exhibit anterior segment defects and the absence of the meibomian gland,[8] further implicating the role of EGFR signaling during anterior segment and meibomian gland development. Furthermore, overexpression of either *Tgfα* or *Egfr* in the lens results in the failure of corneal mesenchymal cells to differentiate into corneal endothelial cells, thus leading to severe anterior segment defects.[41] It has been shown that TGFα can chemoattract migrating mesenchymal cells[42] that lead to the formation of the corneal stroma, corneal endothelium, and associated drainage structures.[43] The role of EGFR during meibomian gland development is unclear. Meibomian glands form around P0.5 from the differentiating epithelial cells near the inner surface of the lid margin.[7] Proper anterior segment development, as well as proper meibomian gland development observed in *Egfr*^{Dsk5} rescued *woe* mice, may be due to rescued ADAM17-mediated EGFR function in these two tissues. Alternatively, the normal ocular and meibomian gland phenotypes in *Egfr*^{Dsk5} rescued *woe* mice may simply be a result of the rescued eyelid closure defect. The role of the ADAM17-mediated EGFR signaling during anterior segment and meibomian gland development at this point still remains elusive and requires further clarification.

Acknowledgments

The authors thank Benjamin Feiner at the Medical College of Wisconsin for his assistance with the genotyping of *woe* and *Dsk5* alleles, and Doug Holmyard at the Mount Sinai Hospital, Toronto, Canada for his assistance with scanning electron microscopy.

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